Defining the role of the transcription factor T-bet in the innate immune system in intestinal inflammation.

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DEFINING THE ROLE OF THE TRANSCRIPTION FACTOR T-BET IN THE INNATE IMMUNE SYSTEM IN INTESTINAL INFLAMMATION

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Abstract

Mice lacking the transcription factor T-bet in the innate immune system (*Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ Ulcerative Colitis, or TRUC) develop microbiota-dependent inflammatory bowel disease (IBD). The innate immune mechanisms responsible for causing disease are still to be resolved, yet potentially offer novel insights into the pathogenesis of IBD, and how T-bet might influence inflammation in the gut.

In this thesis it was shown that chronic IBD in TRUC mice was dependent on interleukin-17A (IL-17A) producing IL-7R^{+} CD90^{+} innate lymphoid cells (ILCs). Depletion of ILCs or IL-17A neutralization cured disease. Cytokines responsible for driving innate IL-17A included IL-23, IL-6 and TL1A. IL-23 and IL-6 played functionally important roles in TRUC disease, since neutralisation reduced innate IL-17A production and attenuated disease. TNFα, predominantly produced by CD11c^{high} class II^{+} CD11b^{+} CD103^{-} dendritic cells (DCs), synergised with IL-23 to drive IL-17A production by ILCs.

Helicobacter typhlonius (HT) was identified as the key colitigenic bacterium driving TRUC disease. Four-five-four ribosomal RNA gene sequencing demonstrated that HT was consistently present in the faeces of all TRUC mice, but absent from a newly-derived colony of disease-free *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ mice. Inoculation of pure HT cultures was sufficient to reinstate disease into this new colony of healthy mice.

T-bet impacted on colitis in several ways. In the absence of T-bet, ILCs selectively produced IL-17A and were poor producers of interferon-γ. Second, T-bet deficient DCs produced excess TNFα in comparison with T-bet sufficient DCs. Finally, T-bet was a transcriptional repressor of the *Il7ra* locus. IL-7R was expressed by ILCs and signalling through this receptor was critical for TRUC disease. Selective IL-7R blockade attenuated disease. Together these data provide new insights into TRUC disease and demonstrate at least some of the mechanisms by which T-bet regulates the interplay between mucosal innate immune cells and the intestinal microbiota.

This thesis is dedicated to

Suzy Powell, Wilfred Powell and Shirley Powell.

List of Contents

List of Tables

List of Figures

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List of abbreviations

AhR	Aryl hydrocarbon receptor
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation or Crohn's disease
ChIP	Chromatin immunoprecipitation
cLP	Colonic lamina propria
DC	Dendritic cell
DSS	Dextran sodium sulphate
DTR	Diphtheria Toxin Receptor
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
AIEC	Adherent and invasive *Escherichia coli*
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FoxP3	Forkhead Box P3
GATA-3	GATA-binding protein 3
GC	Glucocorticoid
GFP	Green fluorescent protein
GWA	Genome wide association (study)
H&E	Haematoxylin and eosin
Hh	*Helicobacter hepaticus*
HT	*Helicobacter typhlonius*
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease

IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
i.p.	Intraperitoneally
IQR	Inter quartile range
Lin^-	$Lineage^-$ (negative)
LP	Lamina propria
LPL	Lamina propria leukocytes
LTi	Lymphoid tissue inducer (cell)
mAb	Monoclonal antibody
MDP	Muramyldipeptide
MFI	Mean (median) fluorescence intensity
MHC	Major histocompatibility complex
mLN	Mesenteric lymph nodes
MNV	Murine norovirus
ND	Not detected
NK	Natural killer
NKT	Natural killer T cell
NOD	Nucleotide-binding oligomerisation domain-containing proteins
OTU	Operational taxonomic unit
PAMP	Pathogen associated molecular patterns
PRR	Pattern recognition receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches

Rag1	Recombination activating gene 1
Rag2	Recombination activating gene 2
mRNA	Messenger ribonucleic acid
RORγt	Retinoic acid related-orphan receptor γt
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SEM	Standard error of the mean
SFB	Segmented filamentous bacteria
siRNA	Small interfering RNA molecules
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TAE	Tris-acetate-EDTA
T-bet	T-box expressed in T cells
Tbx21	T-box transcription factor 21 (gene encoding T-bet)
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNFα	Tumour necrosis factor-α
T-reg	Regulatory T-cell
TRUC	*Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ Ulcerative Colitis
UC	Ulcerative colitis
WT	Wild type
16S	16S subunit of rRNA

CHAPTER 1

Introduction

The mucosal immune system is responsible for policing the epithelial barrier surfaces. The importance of this task is apparent when considering the global impact of infections affecting the gut, lungs and urogenital tracts. However, inappropriate activation of mucosal immunity directed against commensal organisms with which we share our barrier surfaces may lead to collateral damage to host tissue. The challenge of maintaining this delicate balance is perhaps best demonstrated in the gut, where the highest volume of commensal bacteria reside. Although gastrointestinal infections are a leading cause of death globally and robust immunity against luminal pathogens must be maintained, overly exuberant immune responses deployed against commensal organisms is thought to result in collateral damage to the gut and the emergence of an increasingly common immune mediated disease, termed inflammatory bowel disease (IBD).

Recognition and response to the microbial communities occupying the gastrointestinal tract by the mucosal immune system is now recognised to play a central role in both the maintenance and loss of intestinal homeostasis. Insights into the molecular mechanisms regulating mucosal immunity at the interface with the intestinal microbiota are needed to help understand IBD. Transcription factors are proteins that selectively switch genes on or off, enabling specific cells, including immune cells, to perform particular functions, including inflammatory responses. The transcription factor T-bet is expressed by several immune cells and regulates the inflammatory behaviour of these cells. Mice genetically lacking T-bet in the innate immune system spontaneously develop microbiota-dependent intestinal inflammation that resembles IBD and elucidating the immune mechanisms responsible for causing disease in these mice offers the opportunity to gain novel insights into IBD pathogenesis. In this thesis specific hypotheses were tested regarding possible mechanisms of disease in T-bet deficient mice in order to advance our understanding of how T-bet impacts on host-microbe interactions in the gut and to shed new light on possible mechanisms of disease in IBD.

1.1 Inflammatory bowel disease

IBD describes a group of conditions characterised by chronic inflammation of the gastrointestinal (GI) tract. The 2 main forms of the disease are Crohn's disease (CD) and ulcerative colitis (UC). CD can affect any part of the GI tract, but UC is restricted to the colon (Baumgart and Sandborn, 2007). Both forms follow a relapsing and remitting clinical course and commonly experienced symptoms include diarrhoea, rectal bleeding, faecal urgency, tenesmus and abdominal pain (Baumgart and Sandborn, 2007). Extra-intestinal manifestations are also recognised affecting the joints (Orchard *et al*., 1998), eyes (Mintz *et al*., 2004), skin (Lebwohl and Lebwohl, 1998), and hepatobiliary system (Raj and Lichtenstein, 1999). In addition to causing distressing symptoms IBD is associated with significant complications, including venous thromboembolism (Miehsler *et al*., 2004; Solem *et al*., 2004), anaemia (Goodhand *et al*., 2011) malnutrition (Mijac *et al*., 2010; Valentini *et al*., 2008) and colorectal cancer (Eaden *et al*., 2001; Rutter *et al*., 2006). Approximately 80% of patients with CD will require intestinal surgery in their lifetime and about 10-30% of UC patients will ultimately require colectomy (Cosnes *et al*., 2011). IBD is linked to excess mortality (Bewtra *et al*., 2013).

In view of this burden of disease it is unsurprising that IBD negatively impacts on the quality of life and psychosocial wellbeing of affected patients. Chronic fatigue (Jelsness-Jorgensen *et al*., 2011), anxiety and depressive disorders (Graff *et al*., 2009), lost work days (Boonen *et al*., 2002), unemployment (Bernstein *et al*., 2001; Boonen *et al*., 2002) and difficulties with interpersonal and sexual relationships (Trachter *et al*., 2002) are reported with increased frequency in IBD patients. In Europe and North America the incidence of CD and UC is between 0.7-9.8 and 1.5-20.3 cases per 100,000 person-years, respectively (Loftus, 2004), and over the last 50 years the incidence of IBD appears to be increasing (Cosnes *et al*., 2011).

The aetiology of IBD is unknown. However, both genetic and environmental factors are believed to contribute to disease. Twin studies from Northern Europe show concordance rates among monozygotic twins with CD of 35-64%, and in UC between 16-19%. In contrast, concordance between dizygotic twins is just 2-5% in CD and 0-2% in UC (Halfvarson *et al*., 2003; Jess *et al*., 2005; Spehlmann *et al*., 2008; Tysk *et al*., 1988). These data confirm that in most patients neither genetic nor

environmental factors by themselves are sufficient to account for IBD, but instead it is likely that combinations of these factors contribute to disease development.

1.1.1 Genetic insights into IBD pathogenesis

In the last 10 years considerable advances have been made in our understanding of the role of genetics in IBD. In the main these advances have been facilitated by the advent of genome wide association (GWA) studies, in which the profile of many thousands of single nucleotide polymorphisms (SNPs) are compared between groups of disease defined cases and disease free controls. The first major GWA study in IBD was reported in 2007 in a landmark study by the Wellcome Trust case control consortium that compared the distribution of SNPs in 7 common diseases, including Crohn's disease (Wellcome Trust Case Control Consortium, 2007). One advantage of GWA studies is that they provide unbiased, non-hypothesis driven profiles of the genetic architecture of individuals with specific diseases, with the potential to uncover novel biological pathways involved in mediating disease. Currently, polymorphisms at 163 genetic loci are significantly associated with altered IBD risk, including genes encoding proteins involved in immune cell activation, cytokine signalling, microbe recognition and intestinal epithelial barrier function (Jostins *et al.*, 2012). Most IBD susceptibility genes are shared between CD and UC, and indeed with other immune mediated diseases, such as ankylosing spondylitis and psoriasis. IBD genetic susceptibility also overlaps with infectious diseases and immunodeficiency syndromes (Jostins *et al.*, 2012). Genetic studies have provided some novel insights into hitherto unsuspected immune pathways that are now regarded as potentially disease relevant pathways in IBD. Examples include NOD2, autophagy pathway proteins and components of the IL-23/IL-17 cytokine axis.

As early as 1996, before the advent of GWA studies, the first Crohn's disease susceptibility locus was identified on chromosome 16 by studying the genes of families with multiple affected members (Hugot *et al.*, 1996). Subsequently, two independently conducted linkage analysis studies were reported simultaneously as back to back articles in Nature identifying that the gene responsible for conferring disease susceptibility on chromosome 16 encoded a protein called NOD2. Interestingly, NOD2 is an intracellular sensor of bacterial products and like several toll-like receptors (TLRs) is involved in the innate immune response. The specific

ligand of NOD2 is a bacterial cell wall product called muramyl dipeptide (MDP). In one of the initial papers from 2001, one of the susceptibility mutations identified at the *NOD2* locus was located in sequences encoding a functionally important part of the protein termed the leucine-rich repeat domain, which is now recognized as the portion of the molecule responsible for ligand interaction (Hugot *et al*., 2001; Ogura *et al*., 2001). Ogura *et al.* showed that in contrast to the WT protein, the disease variant forms of NOD2 had impaired activation of the pro-inflammatory transcription factor NFκB in response to stimulation with TLR agonists, indicating that *NOD2* mutations were loss of function mutations. Additional functional studies by van Heel *et al*. showed that PBMCs from Crohn's disease patients with the most common *NOD2* mutations had impaired activation in response to direct stimulation with MDP (van Heel *et al*., 2005). In patients with *NOD2* mutations there was impaired MDP induced production of IL-8 by PMBCs, but also impaired release of other crucial pro-inflammatory cytokines when PBMCs were co-stimulated with MDP and other TLR agonists. As well as being the most strongly associated risk allele in Crohn's disease, NOD2 is notably absent from the known list of genes that alter susceptibility to UC, indicating that there are indeed differences in the genetic architecture of CD and UC.

In addition to confirming *NOD2* as a disease susceptibility locus, GWA studies have identified numerous other genes involved in innate immunity. Crohn's disease is associated with SNPs at loci encoding the autophagy pathway components *ATG16L1* and *IRGM* (Jostins *et al*., 2012). The role of autophagy in Crohn's disease has yet to be fully resolved, but the identification of these loci as susceptibility alleles reinforces the notion that innate immune handling of intestinal bacteria is likely important in IBD. There appear to be interactions between autophagy and NOD2. Stimulation of NOD2 with MDP has been shown to trigger autophagy in DCs, and patients with risk variant *NOD2* or *ATG16L1* alleles have defective autophagy and impaired antigen-presentation to T-cells (Cooney *et al*., 2010). These data are important because they show that risk variant alleles at different loci affecting different genes in IBD potentially interact to affect immunological processes in the gut. They show that genetic variation in innate immune pathways may subsequently impact adaptive immune responses. Like *NOD2*, polymorphisms at loci encoding autophagy components were initially thought to be restricted to Crohn's disease.

However, a recent meta-analysis, which has combined data from most of the large IBD GWA studies completed to date, comprising 75,000 cases and controls, has shown that although SNPs at the *ATG16L1* locus are confined to patients with Crohn's disease, risk variant polymorphisms at the *IRGM* locus are also shared with UC.

One of the other most notable immunological pathways linked to IBD through GWA studies is the IL-23/IL-17 axis, comprising the innate immune cytokine IL-23 and the effector cytokine IL-17A. SNPs at a number of components involved in this pathway are significantly associated with IBD, including *IL12B* (one of the IL-23 subunits), *IL23R* (its specific receptor), and the IL23R signalling components *STAT3*, *JAK2* and *TYK2* that are activated by ligation of the IL-23R with IL-23. In addition, SNPs at loci encoding the transcription factor *RORC* and the chemokine receptor *CCR6*, which are markers associated with Th17 cells, are also significantly associated with IBD, and notably, these polymorphisms are shared between UC and CD (Jostins *et al*., 2012).

Other genetic insights into immune pathways potentially relevant in IBD have been gained from reports of patients with extreme phenotypes of intestinal inflammation, or IBD-like syndromes, using genetic linkage analysis and candidate gene sequencing. A case series has been described of children born to consanguineous parents, who developed severe, early onset intestinal inflammation resembling CD. The children had mutations in the genes encoding a subunit of the IL-10 receptor, which resulted in impaired receptor signalling (Glocker *et al*., 2009). IL-10 is recognised as an immunomodulatory cytokine, which inhibits the production of inflammatory cytokines, such as TNFα. PBMCs from the children with loss of function mutations at the *IL10R* locus produced excess TNFα (Glocker *et al*., 2009). Mutations at the *IL10* locus are also associated with IBD in GWAS, corroborating the potential relevance of this pathway (Jostins *et al*., 2012).

In summary, IBD is now among the most complex genetic diseases known and is associated with polymorphisms at more genetic loci than any other disease. Many of the genes implicated in IBD are immune related genes, encompassing both innate and adaptive immune processes and processes involved in immune handling of bacteria are strongly implicated, especially in CD. In addition, cytokine responses

typically deemed to be important in adaptive immune responses, such as the IL-23/IL-17 axis, also appear to play a role.

1.1.2 Environmental considerations in IBD

Since there is only partial concordance between monozygotic twins (Tysk *et al*., 1988), and IBD incidence appears to be increasing over a relatively short period of time (i.e. far too quickly to be accounted for by genetic evolution), it follows that non-genetic factors contribute to IBD risk. Environmental factors associated with altered IBD risk include smoking, geography, exposure to infections and whether individuals have had operations, such as appendectomy or tonsillectomy. In most cases the causal relationships between environmental exposure and IBD risk have still to be resolved.

It has long been recognised that UC is negatively associated with smoking. In 1982 Harries *et al*. conducted a large questionnaire of IBD and control patient, which showed that only 8% of UC patients were current smokers in comparison with 44% of healthy controls (Harries *et al*., 1982). As well as replicating the findings of the earlier study, other studies have shown that CD patients are more likely to be smokers (Somerville *et al*., 1984; Tobin *et al*., 1987). In CD, smoking is also associated with an increased risk of disease relapse, particularly in women (Sutherland *et al*., 1990).

There are data from across Europe comparing IBD risk in patients who have previously undergone tonsillectomy or appendectomy. In the main these studies tend to show reduced UC risk in patients who have previously had a tonsillectomy or appendectomy, whereas these operations tend to confer increased risk of CD (Castiglione *et al*., 2012; Gearry *et al*., 2010; Hansen *et al*., 2011; Russel *et al*., 1997). There are confounding issues that potentially complicate the interpretation of these epidemiological studies. For example, an initial presentation of terminal ileal CD could easily be confused clinically with appendicitis. Therefore, increased incidence of CD in patients who have had an appendectomy may merely reflect patients presenting for the first time with CD were mistakenly diagnosed with appendicitis and underwent appendectomy, only to re-present later with CD.

Dietary factors have also been linked to IBD development. High sugar consumption is associated with increased IBD risk, whereas increased consumption of vegetables and whole meal bread may be protective (Hansen *et al*., 2011; Jakobsen *et al*., 2013). Although there is a vast literature on the potential role of omega-3 fatty acids (fish oils) in IBD, particularly CD, meta-analyses evaluating trials of omega-3 supplementation in CD do not show therapeutic benefit (Turner *et al*., 2009).

Another intriguing environmental factor that appears to predispose to IBD development is exposure to gastrointestinal infection (Garcia Rodriguez *et al*., 2006; Hansen *et al*., 2011; Porter *et al*., 2008), including well documented cases of microbiologically proven *Salmonella* and *Campylobacter* gastroenteritis (Gradel *et al*., 2009). There is a growing literature concerning the role of pathogenic organisms, pathobionts and even commensal bacterial species in the breakdown of intestinal homeostasis and the emergence of chronic intestinal inflammation. Indeed, the profile and community structure of the intestinal microbiota is now regarded as a key environmental variable responsible for influencing host health.

1.2 Host microbe interactions at the epithelial barrier surfaces

Following birth the epithelial barrier surfaces of mammals become permanently colonised with prodigious numbers of microorganisms, including an estimated 10^{12} bacteria colonising the skin and 10^{14} bacteria occupying the GI tract of humans (Luckey, 1972). Until recently defining the community composition of the microbiota colonising mammalian hosts has been hampered by difficulties in cultivating bacteria from certain body habitats. However, following the emergence of culture independent molecular techniques it is now possible to accurately determine the profile and frequency of microbes occupying particular ecological niches. These unbiased approaches rely upon sequencing of conserved regions within the 16S rRNA genes of microbes using region specific primers and high-throughput DNA sequencing. These techniques have provided crucial new insights into our understanding of the microbes occupying our epithelial barrier surfaces. We now know that the composition of the microbiota is highly variable, both between individuals as well between different body habitats in the same individual (Huttenhower *et al*., 2012; Eckburg *et al*., 2005). The Human Microbiome Project Consortium has recently published detailed data regarding the community composition of the microbiota colonising 18 body habitats, including the skin, urogenital and GI tracts of 242 healthy adults (Huttenhower *et al*., 2012). In this landmark study the community profile of the major bacterial taxa was distinct and characteristic at different anatomical sites. Some niches, such as the saliva, harboured a diverse range of operational taxonomic units (OTUs) with little variation between study individuals, whereas the skin contained fewer overall species but with much greater variation between individuals. This study also highlighted how particular phyla tend to favour environmental conditions created by particular body habitats. For instance, the posterior fornix of the cervix is almost totally dominated by *Firmicutes*, whereas the anterior nares are mostly composed of *Actinobacteria*. The Human Microbiome Project has largely corroborated data from earlier studies with fewer subjects (Costello *et al*., 2009), and has also established that these microbial communities are largely stable over time.

As well as harbouring the greatest total number of organisms, the microbiota of the distal GI tract is also one of the most diverse in terms of community membership and inter-individual variation (Huttenhower *et al*., 2012; Costello *et al*., 2009; Eckburg *et al*., 2005). The vast majority of organisms present are bacteria with minor contributions from archaea (0.8%), other non-human eukaryotic cells (0.5%) and viruses (up to 5.8%) (Arumugam *et al*., 2011). At the phylum level *Bacteroidetes* and *Firmicutes* represent >90% of bacteria present in the distal GI tract of humans (Eckburg *et al*., 2005), mice (Ley *et al*., 2005) and indeed other mammals (Ley *et al*., 2008). Collectively in humans over 1,000 bacterial species have been identified in this habitat and each individual harbours approximately 160 different bacterial species (Qin *et al*., 2010). Intestinal archaea are much less diverse with one species in particular, *Methanobrevibacter smithii*, dominating (Eckburg *et al*., 2005; Gill *et al*., 2006). Virus-like particles seem to be a stable but highly individual trait, with minimal influence of either family or environment (Reyes *et al*., 2010). The composition of the microbiota occupying the lower GI tract mucosa is also distinct from microbiota present in the faecal stream (Eckburg *et al*., 2005), indicating that there is compartmentalisation of the microbial communities colonising the gut. The composition of the intestinal microbiota is also recognised to vary according to geography and age (Yatsunenko *et al*., 2012). Although neonates are born germ free they are immediately colonised by maternally derived bacteria that they are exposed to during birth. Vaginally delivered babies are rapidly colonised at different body habitats by taxa present in the mother's vagina, including *Lactobacillus*, *Prevotella* and *Atopobium*, whereas infants delivered by caesarean section are predominantly colonised by skin taxa, such as *Staphylococcus* spp (Dominguez-Bello *et al*., 2010). However, by 3 years of age the composition of the intestinal microbiota largely evolves to resemble that of adults (Yatsunenko *et al*., 2012), demonstrating that the environmental conditions of a particular ecological niche strongly influence the ultimate success of microbial colonisation. Marked variation in the composition of intestinal microbiota in individuals from different geographical and socioeconomic backgrounds is well recognised (De Filippo *et al*., 2010; Yatsunenko *et al*., 2012).

Importantly, the organisms with which we share our epithelial barrier surfaces seldom cause disease in immunologically competent individuals and instead they often confer survival advantage to the host, by performing a variety of beneficial

functions (Figure 1). Intestinal bacteria possess enzymes genetically absent from mammals enabling their host to utilise novel metabolic pathways. These bacteria salvage nutrients for the host by degrading otherwise indigestible complex dietary polysaccharides (Xu *et al*., 2003), and generating utilisable short chain fatty acids, such as butyrate and acetate by fermentation (Mahowald *et al*., 2009). The microbiota also perform protective roles for the host, including detoxification of xenobiotics (Swann *et al*., 2009) and limit colonization by invasive pathogens, such as *E.coli* 0157:H7 (Asahara *et al*., 2004).

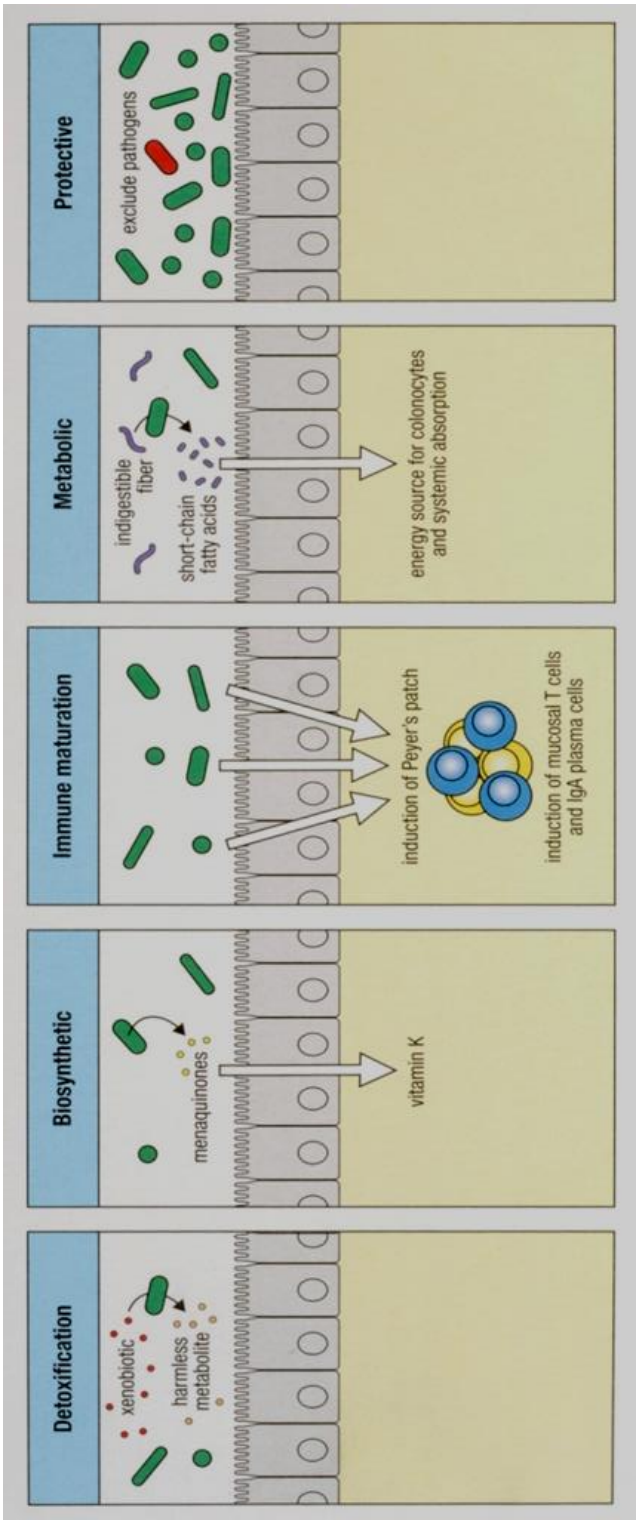

Figure 1. **The intestinal microbiota confers health benefits to the host.** Ingested xenobiotics are metabolised into inert by-products. Biosynthetic functions involve synthesis of biomolecules utilisable by the host (e.g. microbial enzymes biosynthesize vitamin K). Immune maturation is dependent on the microbiota (see Table 1). Metabolic functions include fermentation of indigestible dietary fibres into metabolites utilisable by the host (e.g. short-chain fatty acids). Microbes provide protection by competing with pathogens for space and nutrients. Adapted from Powell *et al*., 2013.

1.2.1 Host perception of the microbiota

For the host/microbiota symbiosis to flourish the host must be able to sense and react to the microbes to which it is exposed at the barrier surfaces, but must also discriminate between pathogens and symbionts. This difficult task is perhaps most challenging in the GI tract, since this location houses the greatest number of symbionts but is also one of the sites most susceptible to invasion by pathogens, and worldwide GI infection remains one of the most important causes of morbidity and mortality (Motarjemi and Kaferstein, 1997). The microbiota is sensed by cells of the intestinal mucosa, including epithelial cells, macrophages, DCs, endothelial cells, myofibroblasts and adipocytes (Cario, 2005). These cells are equipped with a diverse repertoire of receptors, termed pattern recognition receptors (PRRs) that recognise conserved microbial components termed pathogen associated molecular patterns (PAMPs). Typical PAMPs include bacterial cell wall molecules, such as lipoteichoic acid, peptidoglycans and lipopolysaccharide (Cario, 2005). The best known PRRs are the TLRs. These transmembrane receptors share common structural motifs and at least 11 family members have been identified in mammals (Cario, 2005). Another structurally distinct family of PRRs is the NOD like family of receptors, which are intracellular receptors (Cario, 2005). By controlling the repertoire, cellular distribution and tissue localisation of PRRs the host can sense and respond to environmentally encountered microbes in different ways in different anatomical locations. For instance, TLR expression may be constitutive or induced, and maybe differentially expressed on the apical or basolateral surface of the mucosal epithelium (Cario, 2005). Therefore, luminal facing TLRs expressed on the apical surface of the epithelium directly interface with colonic microbes, whereas those expressed on the basolateral aspect occupy a “privileged” compartment that in the healthy state is not exposed to luminal microbes. For example, TLR5 is preferentially expressed on the basolateral aspect of intestinal epithelial cells (Gewirtz *et al*., 2001), where it is denied access to its specific agonist flagellin, a conserved structural protein forming the flagella of numerous bacteria (Hayashi *et al*., 2001). However, bacterial translocation occurring following epithelial damage in acute GI infection with flagellated pathogens, such as *Salmonella*, results in *de novo* exposure of TLR5 to its ligand and induction of a protective pro-inflammatory cytokine response (Gewirtz *et al*., 2001). Homozygous loss of function mutations in the TLR5 gene results in

increased susceptibility to Legionnaire's disease, a severe lung infection caused by another flagellated bacteria *Legionella pneumophilia* (Hawn *et al*., 2003).

Despite their role in stimulating anti-pathogen immunity, it is also recognised that PRRs interact with gut symbionts to facilitate their mutualistic relationship with the host. The critical importance of the cross talk between the innate immune system and the gut microbiota for the maintenance of intestinal homeostasis is readily demonstrated by experimental perturbation of either of these components. Disrupting innate immune recognition of luminal organisms, for example in mice with genetic deficiencies in particular PRRs results in disordered intestinal homeostasis and altered susceptibility to inflammatory insult. Indeed, genetic deletion of MyD88, the adaptor molecule responsible for TLR ligation mediated induction of NFκB, results in enhanced susceptibility of mice to DSS-induced colitis (Rakoff-Nahoum *et al*., 2004). *Myd88*$^{-/-}$ mice have exaggerated turnover of intestinal epithelial cells and are unable to generate protective factors which promote epithelial homeostasis as a consequence of their inability to receive signals from the commensal intestinal microbiota. Commensal bacteria were responsible for TLR activation since robust depletion with potent mixtures of antibiotics also rendered WT mice highly susceptible to DSS colitis/mortality. This sensitivity to disease could be rescued by treating microbiota-depleted mice with purified TLR agonists LPS and LTA (which ligate TLR4 and TLR2, respectively). In agreement with these findings *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice are also highly sensitive to DSS-induced colitis/mortality, which cannot be rescued by administration of LPS (in the case of *Tlr4*$^{-/-}$ mice) or LTA (in the case of *Tlr2*$^{-/-}$ mice) following microbiota depletion (Rakoff-Nahoum *et al*., 2004). Together these data show that signals delivered by the normal intestinal commensal microbiota play an important role in intestinal homeostasis.

Similarly, Mice lacking TL9 have a pro-inflammatory phenotype and adoptive transfer of colonic T-cells from *Tlr9*$^{-/-}$ mice to immunodeficient recipients triggers more severe colitis than transfer T-cells from the gut of WT mice (Hofmann *et al*., 2014). Mice lacking TLR5 develop spontaneous colitis (Vijay-Kumar *et al*., 2007). Taken together these data demonstrate that the role of immune recognition of luminal commensal bacteria is a highly complex process.

Studies of germ-free mice have also provided useful insights into the importance of the intestinal microbiota in immune homeostasis. Mice reared in sterile conditions devoid of exposure to environmental microbes have numerous immunological, physiological and anatomical differences from conventionally reared animals (Table 1). These changes include grossly enlarged caeca, poor development of gut associated lymphoid tissue, fewer mononuclear cells infiltrating the lamina propria and reduced TLR expression (Thompson and Trexler, 1971; Wang *et al*., 2010).

Table 1. Abnormalities apparent in germ-free mice. Adapted from Powell *et al*., 2013.

Anatomical/histological	Immunological	Functional
Enlarged cecum	Reduced secretory IgA production and low level of serum immunoglobulin	Increased susceptibility to infections: • *Salmonella* • *Listeria* • *Shigella* • *Leishmania* • *Bacillus anthracis*
Longer small intestine with taller villi in the duodenum and shorter villi in the ileum	Diminished numbers and activation of systemic T cells	
Poorly developed mesenteric lymph nodes	Reduced $CD8^+$ T-cell cytotoxicity	Reduced susceptibility to autoimmune disease: • Experimental allergic encephalomyelitis • Arthritis • Some models of inflammatory bowel disease
Poorly developed Peyer's patches	Impaired lymphocyte homing to inflammatory sites	
Lower numbers of isolated lymphoid follicles	Reduced intestinal lamina propria lymphocytes and intraepithelial lymphocytes (IELs), especially αβ T-cell receptor IEL	
	Impaired helper T cell T_H17 T-cell responses	
Small spleen	Reduced ability of granulocytes to kill bacteria	Reduced ability to induce oral tolerance

1.2.2 Host immunity is modulated by the microbiota and impacts on host health

The intestinal microbiota profoundly impacts on key aspects of host immunity. Unlike conventionally colonised mice, there are few T cells present in the intestinal lamina propria and intraepithelial compartment of germ free mice (Okada *et al*., 1994). T-cell dependent delayed type hypersensitivity reactions are also impaired in germ free animals (Carter et al., 1979; Collins and Carter, 1980). The differentiation of effector $CD4^+$ T-cell lineages has also been shown to be modulated by the community composition of the intestinal microbiota. It was recently recognised that the capacity of a single strain of mice to generate Th17 responses was dependent on which commercial vendor supplied the mice. C57/B6 mice supplied by Jackson laboratories, the largest vendor of research mice in the United States, were poor generators of IL-17 responses, whereas mice acquired from Taconic Farms and a number of other vendors readily produced IL-17 (Ivanov *et al*., 2008). These data indicate that in addition to host genetics, environmental factors also play a central role dictating host capacity to mount effector T-cell responses. Crucially, the intestinal microbiota was identified as the key environmental variable responsible for influencing Th17 differentiation, since Th17 responses could be restored in Jackson mice if they were co-housed with Taconic Farms mice or if they were gavaged with the caecal contents harvested from Taconic Farms mice (Ivanov *et al*., 2008). Similarly, a potent cocktail of 4 different antibiotics that has been shown to dramatically reduce the microbial load present in the intestine also resulted in diminished Th17 differentiation (Ivanov *et al*., 2008). For the first time, these studies demonstrated that the microbial communities interfacing with the host immune system helps to shape T-cell lineage commitment. 16S rRNA gene sequencing identified differences in the intestinal microbiota communities between Jackson and Taconic Farms mice, including the identification of an unculturable bacterium from the *Arthomitus* genus termed segmented filamentous bacteria (SFB) that was present in Taconic farms, but not Jackson mice (Ivanov *et al*., 2009). Crucially, monoassociation of re-derived germ free mice from Jackson with SFB but not other intestinal bacteria resulted in successful acquisition of Th17 responses (Ivanov *et al*., 2009). In addition to driving Th17 responses, it has also been demonstrated that SFB may be important in driving Th1 immunity (Gaboriau-Routhiau *et al*., 2009). SFB

colonises the terminal ileum where it tightly adheres to the ileal mucosa and penetrates into epithelial cells (Ivanov *et al*., 2009). The close contact formed between long filaments from SFB and ileal epithelial cells and PPs, a key inductive site of effector T-cells, may be important in their capacity to modulate T-cell immunity. Analysis of the gene expression profile of the ileum following colonisation of germ free mice with SFB demonstrated upregulation of many genes involved in host immunity, such as the antimicrobial peptide RegIII and acute phase proteins, such as serum amyloid A, which has been shown to promote Th17 differentiation *in vitro* (Ivanov *et al*., 2009).

Modulation of host immunity by the intestinal microbiota is also functionally relevant in host health. Germ free mice are more susceptible to infections, such as *Listeria monocytogenes* (Inagaki *et al*., 1996), *Nocardia asteroids* (Beaman *et al*., 1980) and *Cryptosporidium parvum* (Sacco *et al*., 1998). Paradoxically, germ free mice are less susceptible to T-cell mediated immune diseases, including IBD (Sellon *et al*., 1998), autoimmune arthritis (Wu *et al*., 2010) and experimental allergic encephalomyelitis (EAE), a model of multiple sclerosis (Lee *et al*., 2011). Furthermore, in the case of arthritis and EAE it has been shown that monoassociation of germ free mice with SFB was sufficient to successfully reinstate Th17 responses and restore disease susceptibility (Lee *et al*., 2011; Wu *et al*., 2010). In the GI tract the Th17 response is also important in host resistance to certain pathogens, including *Citrobacter rodentium* (Symonds *et al*., 2009), *Shigella flexneri* (Sellge *et al*., 2010) and *Salmonella typhimurium* (Raffatellu *et al*., 2008). SFB permissive Th17 responses are functionally important in host resistance to infection, since Jackson mice lacking SFB and unable to mount robust Th17 responses are highly sensitive to *Citrobacter rodentium* infection (Ivanov *et al*., 2009). Crucially, inoculation with SFB restored robust Th17 immunity in Jackson mice and bolstered their resistance to infection (Ivanov *et al*., 2009). SFB do not colonise the GI tract of humans (Sczesnak *et al*., 2011), therefore, a role for particular intestinal bacterial species in stimulating effector T-cell differentiation has yet to be established in humans.

The intestinal microbiota also influences the generation of regulatory T-cells (T-regs). Monoassociation of germ free mice with the human commensal *Bacteroides fragilis* promotes the accumulation of IL-10 producing $Foxp3^+$ T-regs in the intestine (Round

and Mazmanian, 2010). A key molecule involved in this process is the capsular polysaccharide A (PSA) since mutant strains of *Bacteroides fragilis* lacking PSA have impaired T-reg induction (Round and Mazmanian, 2010). PSA mediated T-reg induction is functionally relevant. Administration of purified PSA has been shown to augment the number and suppressive capacity of T-regs and to attenuate experimental IBD induced by either *Helicobacter hepaticus* (Mazmanian *et al*., 2008) or TNBS (Round and Mazmanian, 2010). Metagenomic sequencing of the intestinal microbiota of healthy human subjects has demonstrated considerable variation in the prevalence and frequency of *Bacteroides fragilis* (Huttenhower., *et al*. 2012) and studies evaluating T-reg phenotype in patients with and without *Bacteroides fragilis* are eagerly awaited.

1.2.3 The microbiota and inflammatory bowel disease

The intestinal microbiota is now under scrutiny as a potentially important contributor to IBD pathogenesis, and evidence supporting a role for the intestinal microbiota in IBD is shown in Table 2. Patients with IBD have circulating antibodies and T-cells specificities directed against normal constituents of their intestinal microbiota (Duchmann *et al*., 1999; Helphingstine *et al*., 1979; Mow *et al*., 2004). Manipulation of the intestinal microflora may be clinically beneficial in IBD. Under some circumstances, antibiotics have proven efficacy in CD, including the treatment of perianal disease (Bernstein *et al*., 1980; Dejaco *et al*., 2003) and in the prevention of disease recurrence following ileocaecal resection (Rutgeerts *et al*., 1995). Antibiotics are the first line therapy for postoperative UC patients who develop pouchitis (Shen *et al*., 2001). Similarly, probiotics, defined as microbes that are beneficial to health have been shown to maintain remission in UC in some studies (Ishikawa *et al*., 2003; Kruis *et al*., 1997) and are therapeutic in pouchitis (Gionchetti *et al*., 2000). Probiotic bacteria appear to alter the balance between inflammatory and regulatory pathways in the intestine. For instance, the probiotic mixture VSL#3 induces production of the regulatory cytokine IL-10 by DCs and at the same time reduces the expression of pro-inflammatory cytokines, such as IL-12, and co-stimulatory molecules, including CD80 (Hart *et al*., 2004). In some circumstances, such as patients with extensive small intestinal CD, surgical diversion of the faecal stream away from inflamed intestinal segments, thereby reducing exposure to luminal microbes, is recognised to promote mucosal healing (Harper *et al*., 1985).

Table 2. Evidence implicating the intestinal microbiota as an important environmental variable in IBD. Adapted from Powell *et al.*, 2013.

	Crohn's disease	Ulcerative colitis
Antibiotics useful?	Effective in management of fistulae Effective in penetrating disease with abscess formation Reduces postoperative recurrence	Effective in pouchitis
Probiotics useful?	Not helpful	Effective in pouchitis Effective in maintaining remission
Prebiotics useful?	Not helpful	Yet to be established, likely small/no benefit
Diversion of fecal stream?	Helpful	Not routinely performed for ulcerative colitis
Disease location	Most common location is ileo-cecal disease (areas of high microbe exposure)	Colon only (occasional 'backwash ileitis')
Adherent-invasive *E. coli*	Common	Uncommon
Faecalibacterium prausnitzii	Protective effect	Unknown
T-cell reactivity to microbes	Present	Present
Genetics	Innate immune molecules handling the microbiota (e.g. NOD2)	No innate components implicated to date

The intestinal microbiota also profoundly impact mouse models of intestinal inflammation. *Il10*$^{-/-}$ mice which spontaneously develop colitis when colonised by a conventional intestinal microflora are protected from disease when they are housed under germ free conditions (Sellon *et al*., 1998). Indeed, the penetrance of typhlocolitis in these mice seems to vary according to the cleanliness of the animal facility and some bacteria appear to be particularly effective at triggering disease, such as *Helicobacter hepaticus* (Kullberg *et al*., 1998) and *Enterococcus faecalis* (Kim *et al*., 2005b).

Viral infections have also been implicated in IBD development. Mice with a targeted mutation in the CD associated autophagy gene *Atg16l1* exhibit marked abnormalities of Paneth cells and the intestinal epithelium, including impaired assembly and secretion of antimicrobial peptides and increased expression of pro-inflammatory cytokines, analogous to the mucosal phenotype observed in CD patients with homozygous mutations in *ATG16L1* (Cadwell *et al*., 2008). However, these abnormalities only manifest in mice colonised with a conventional bacterial microflora and concomitant infection with murine norovirus (MNV), which is endemic to many animal houses (Cadwell *et al*., 2010). In WT mice, MNV infection fails to induce abnormalities in either Paneth cells or epithelial cells, demonstrating that interaction between MNV and a specific genetic susceptibility was required to initiate this CD-like mucosal phenotype. MNV infected *Atg16l1* hypomorphic mice were more sensitive to DSS-induced colitis, which resulted in histological features resembling some aspects of CD, including full thickness ileal inflammation with leukocytes infiltrating the muscularis layers and mesenteric fat (Cadwell *et al*., 2010). Furthermore, antibiotic therapy targeting the normal bacterial flora of *Atg16l1* hypomorphic mice, or blockade of the inflammatory cytokines TNFα or IFNγ also attenuated disease, indicating a key role for the interaction between host immunity and intestinal bacteria in disease manifestation (Cadwell *et al*., 2010). Intriguingly, MNV is related to norovirus species that cause acute gastrointestinal inflammation in humans.

Studies have also been undertaken evaluating the community composition of the intestinal microbiota of IBD patients in comparison with non-IBD control subjects using metagenomic sequencing. Several studies have identified quantitative and

qualitative differences in community membership of the intestinal microbiota. A frequently observed difference between IBD patients and healthy control subjects is reduced intestinal community diversity, in both CD and UC patients (Frank *et al*., 2007; Kang *et al*., 2010; Manichanh *et al*., 2006; Ott *et al*., 2004). A relative depletion of members of the *Firmicutes* and *Bacteroidetes* phyla and expansion of *Proteobacteria* and *Actinobacteria* has also been reported in IBD patients (Frank *et al*., 2007). Reduced biodiversity of the intestinal microbiota and relative expansion of *Proteobacteria* and *Actinobacteria* has also recently been observed in IBD affected twins in comparison with their healthy siblings (Lepage *et al*., 2011). It is not certain whether changes in the composition of intestinal microbiota are causal or secondary to inflammation or diarrhoea itself. Reduced intestinal bacterial biodiversity, albeit to a lesser degree, has also been observed in other diarrhoeal illnesses, such as infectious gastroenteritis and irritable bowel syndrome (Mai *et al*., 2006; Noor *et al*., 2010).

Another bacterial component of the intestinal microflora, *Faecalibacterium prausnitzii*, may play an important anti-inflammatory role in the intestine. *F. prausnitzii* is under-represented in patients with active IBD (Sokol *et al*., 2008; Sokol *et al*., 2009), and reduced *F. prausnitzii* frequency is associated with an increased rate of CD recurrence following surgical resection of an inflamed intestinal segment (Sokol *et al*., 2008). *F. prausnitzii* was also observed to attenuate TNBS induced colitis (Sokol *et al*., 2008). Although the mechanisms responsible for the anti-inflammatory properties of *F. prausnitzii* are still to be resolved, it appears that soluble factors contribute at least in part. Supernatants from *F. prausnitzii* cultures significantly inhibited IL-1β induced colonic epithelial production of the inflammatory cytokine IL-8. In contrast to other bacteria, *F. prausnitzii* was a weak inducer of IFNγ and IL-12, and instead promoted production of the anti-inflammatory cytokine IL-10 (Sokol *et al*., 2008), as has been observed with anti-inflammatory probiotic bacterial mixtures such as VSL#3 that are therapeutic in IBD (Hart *et al*., 2004).

Compelling data for a pathogenic role for specific bacterial species in human IBD is lacking. In the early 1980s *Mycobacterium avium paratuberculosis* (MAP) was proposed as a possible aetiological agent in CD (Chiodini *et al*., 1984) and PCR

based technologies initially indicated the presence of this organism in a significant number of intestinal resection specimens from CD, but not UC or non-IBD patients (Sanderson *et al*., 1992). However, other studies have failed to corroborate these findings (Rowbotham *et al*., 1995). Notably, a large meta-analysis of anti-mycobacterial therapy in CD failed to show efficacy (Borgaonkar *et al*., 2000). A subsequent comprehensive prospective study was undertaken in which 213 CD patients were randomised to receive 2 continuous years of anti-mycobacterial therapy (clarithromycin, rifabutin and clofazimine) or placebo, which also failed to show any long term benefit (Selby *et al*., 2007). Therefore, a key role for mycobacterial species in driving IBD seems unlikely.

Other bacterial species have also been implicated in IBD pathogenesis. *Escherichia coli* are enriched in the intestinal mucus layer of CD patients (Martin *et al*., 2004). Moreover, these *E. coli* adhere to and invade intestinal epithelial cells (Boudeau *et al*., 1999). Adherent and invasive *E. coli* (AIEC) attach to specialised membranous/microfold epithelial cells (M cells) that overly Peyer's patches (PPs) through expression of the virulence factors type 1 pili and long polar fimbriae (LPF) (Chassaing *et al*., 2011). Once translocated beyond the ileal epithelium AIEC survive and replicate inside macrophages by evading intracellular killing mechanisms (Glasser *et al*., 2001), thereby facilitating their prolonged access to this key intestinal lymphoid structure that plays such an important role in shaping host intestinal immunity. There have been reports of disordered microbial loading in the intestinal mucus layer in UC. As well as harbouring much higher concentrations of bacteria in the mucus layer than healthy controls the mucus layer of UC patients may also contain potentially pathogenic species (Swidsinski *et al*., 2002). For instance, culture supernatants harvested from *Fusobacterium varum* that were isolated from the colonic mucus layer of UC patients induced colonic ulceration and histological lesions resembling UC when rectally administered to mice (Ohkusa *et al*., 2003).

1.3 Immunology of inflammatory bowel disease

The immunology of IBD is complex, with cross-talk between multiple adaptive and innate immune mechanisms interacting with stromal cells, epithelial cells, endothelial cells and contents of the gastrointestinal lumen, including bacteria and food antigens. A key aspect of diagnosing IBD is histological assessment of mucosal biopsies, which became commonplace following the widespread introduction of fibreoptic colonoscopy and is now routinely performed in clinical practice. In UC there is marked infiltration of the colonic lamina propria with lymphocytes and plasma cells, and usually focal infiltration of neutrophils, which may infiltrate crypts causing their architectural distortion or destruction (Morson, 1972). There is goblet cell depletion and reactive epithelial hyperplasia. Inflammation is usually confined to the mucosa, however, when there is marked destruction of the mucosa, the submucosa may become inflamed. In CD, changes are often patchy and discontinuous, commonly involves the submucosa and beyond (may be full thickness), and tend to cause proportionally fewer crypt abscesses. The infiltrate is mostly lymphocytes with fewer neutrophils, which are found at highest frequency aggregated at ulcers. There also tends to be more oedema and lymphangiectasia in CD in comparison with UC (Morson, 1972; Williams, 1964). The other histological hallmark of Crohn's disease is the formation of epithelioid granuloma. However, granuloma are only present in about one third of CD patients (Heresbach *et al*., 2005). As well as its role in the diagnosis of IBD, histological assessment of IBD confirms involvement of immune cells in disease. The role of different immune cells in IBD has been intensely studied for many years. For context the role of adaptive immunity will be considered in outline only, since the key objective of this thesis is to provide insights into disease relevant innate intestinal immune pathways. Innate immune cells, including a relatively newly described population of innate lymphoid cells will be described in greater depth.

1.3.1 Adaptive immunity and IBD

It has long been recognised that IBD is characterised by increased numbers of activated lymphocytes present in mucosal lesions (Fiocchi *et al*., 1979, 1981). T-cell clones generated from intestinal cLP T-cells isolated from IBD patients are activated,

as measured by proliferative responses, by normal constituents of their gut flora (Duchmann *et al*., 1996; Duchmann *et al*., 1999), indicating that aberrant T-cell activation in IBD is directed against their commensal flora. Initial studies looking at the phenotype of T-cells from IBD patients suggested that CD2/CD28 or CD3/CD38 stimulation was associated with increased IFNγ production in CD and IL-5 production in UC, with the implication that CD is likely a Th1 disease and UC an atypical Th2 disease (Breese et al., 1993; Fuss et al., 1996). Consistent with this, macrophage-derived IL-12, which induces the polarization of naïve T cells towards a Th1 phenotype, is highly up-regulated in inflamed CD but not in UC mucosa (Monteleone *et al*., 1997). IL-12 signals through STAT4, and in CD but not in UC there is abundant phospho-STAT4 in mucosal T cells (Monteleone *et al*., 1997). However, the paradigm of CD as a Th1 disease and UC as an atypical Th2 disease has also been challenged with the recognition that mucosal Th17 responses may play an important role in IBD pathogenesis. It is well established that disease susceptibility genes encoding multiple components of the Th17 pathway are shared between CD and UC (Jostins *et al*., 2012). It is also interesting that susceptibility loci associated with the Th1 response are also shared between CD and UC, including *IFNG*, *STAT1*, *STAT4* and *IL18RAP*, whereas polymorphisms in canonical Th2 genes, including *GATA3*, *IL4*, *IL5* and *IL13* are not associated with UC nor CD (Jostins *et al*., 2012). Other studies looking at differences in T cell-derived cytokines between UC and CD have shown less clear cut distinctions. Th17 cells and Th1/Th17 cells, which release both IFNγ and IL-17A, are increased in the gut of CD and UC patients in comparison with controls, and IL-17A is overexpressed in both CD and UC mucosa (Fujino *et al*., 2003; Rovedatti *et al*., 2009). Transcriptomics is an unbiased way to look for differences in the transcription profile of immune genes in the intestine of patients with CD, UC and healthy controls. Although a number of studies have appeared, and indeed differences have been identified, these are frequently associated with epithelial renewal and basic biochemical processes than with a particular type of immune response (Noble *et al*., 2008; Noble *et al*., 2010; Schulze *et al*., 2008), although dysregulated gene expression of the Th17 components *IL23R*, *STAT3* and *JAK2* was observed in CD (Noble *et al*., 2010).

Treatments targeting T-cells have also shown efficacy in IBD. Cyclosporine, a calcineurin inhibitor that prevents NFAT activation in T-cells, which results in

impaired pro-inflammatory cytokine synthesis (Breese *et al*., 1993) and increased apoptosis (Ying *et al*., 2003), is efficacious, particularly in acute, severe forms of UC that is unresponsive to steroids (Lichtiger *et al*., 1994). Steroids, which are also commonly used to treat IBD, inhibit antigen-induced T-cell cytokine production (Powell *et al*., 2001). However, despite early promise, visilizumab, an anti-CD3 mAb that specifically targets T-cells and is thought to drive T cell tolerance failed to show efficacy in UC and was associated with significant systemic cytokine release limiting its use (Sandborn *et al*., 2010).

T-cells have been shown to play a prominent role in mouse models of IBD. Adoptive transfer of naive T-cells into lymphopaenic mice lacking endogenous lymphocytes induces severe pan-colitis (Powrie *et al*., 1994), and is frequently used by many research groups to investigate mechanisms of disease.

1.3.2 Innate immunity and IBD

Innate immune mechanisms are thought to play an important role in IBD. Mouse models of IBD have been described in mice lacking adaptive immunity, including disease induced by *Helicobacter hepaticus*, DSS and agonistic anti-CD40 mAbs (Buonocore *et al*., 2010; Kim *et al*., 2006; Li *et al*., 1998; Uhlig *et al*., 2006). Innate lineages with important roles in IBD include dendritic cells, macrophages, granulocytes and a new population of cells called innate lymphoid cells, which will be discussed in detail.

1.3.2.1 Innate lymphoid cells

The term innate lymphoid cell (ILC) is collectively used to define functionally heterogeneous cells with shared lymphoid cell characteristics, but lacking adaptive immune receptors. These cells include natural killer (NK) cells and innate populations of cells that express GATA-3, RORγt or T-bet. ILCs lack common lineage markers, such as CD3, CD19, CD11c, CD11b, CD14 and CD16. However, all ILC populations appear to express IL-7R and CD90 (also known as Thy-1). Currently, there is no standardised classification or agreed nomenclature for these populations. Mucosal ILCs can be subdivided into 3 main subsets comprising NK receptor expressing intestinal RORγt$^+$ cells that produce IL-22; GATA-3$^+$ cells that produce Th2 cytokines; and intestinal RORγt$^+$ cells that lack NK receptors and

produce combinations of IL-17, IL-22 and IFNγ. In addition to these mucosal dwelling cells an additional subset of related cells termed lymphoid tissue inducer (LTi) cells are also recognised, that are involved in secondary lymphoid organogenesis. Conventional NK cells involved in systemic immune responses and tumour immunity will not be considered in depth in this thesis. In view of the confusion regarding a standardised classification for ILCs there has been a recent attempt to more formally adopt consistent nomenclature to the different ILC subsets (Spits *et al*., 2013). Group 1 ILCs are defined by their capacity to produce IFNγ, and are subdivided into "conventional" NK cells and ILCs that lack phenotypic traits of NK cells, but possess NK receptors, such as NKp46. These cells express the transcription factor T-bet. Group 2 ILCs are defined by their capacity to produce Th2 cytokines and express GATA-3. Group 3 ILCs produce Th17 cytokines, but are yet further subdivided into LTi like cells, NK receptor^{+} (NKp46^{+}) cells and NK receptor^{-} (NKp46^{-}) populations. This proposed nomenclature is illustrated in Figure 2.

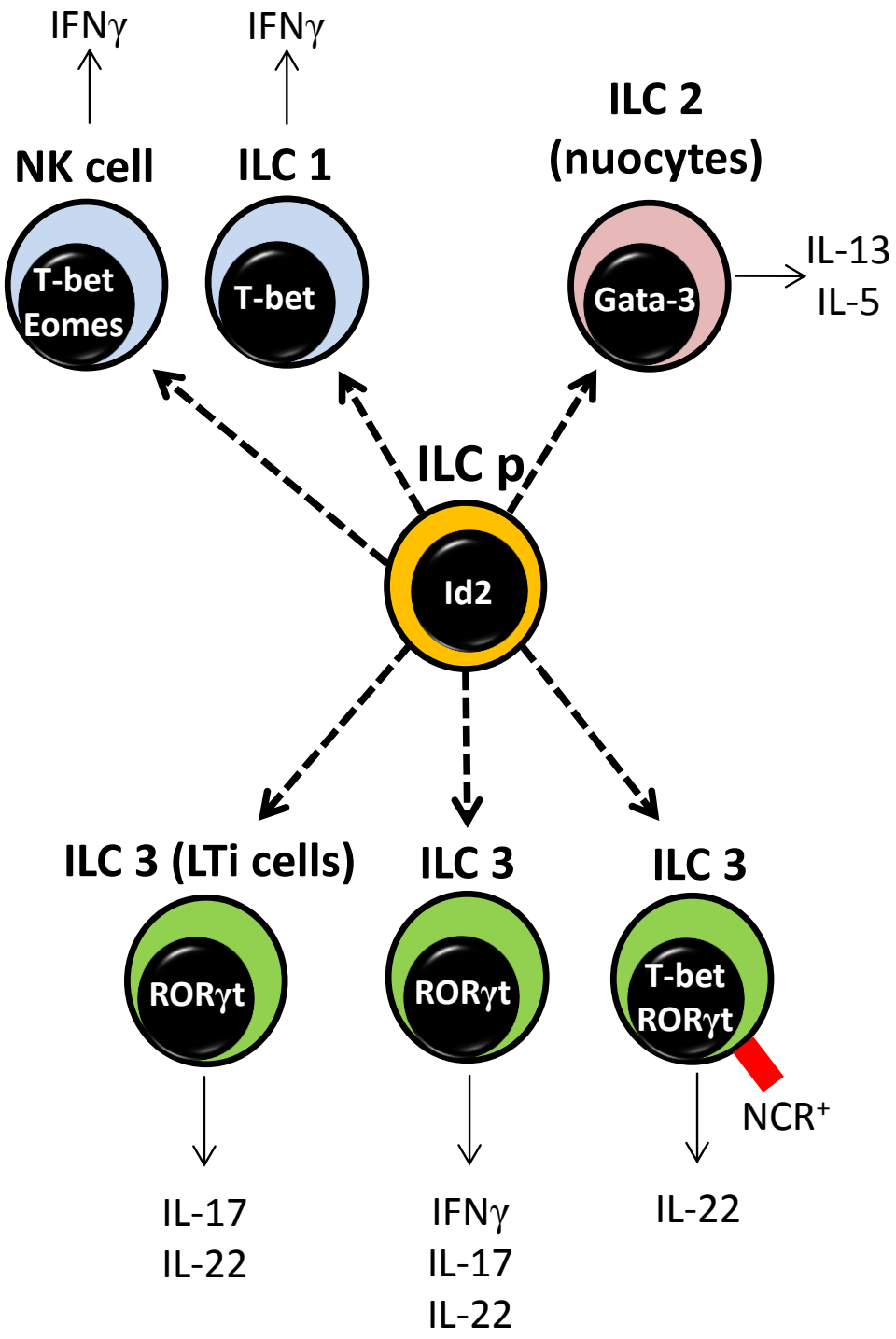

Figure 2. **Proposed nomenclature for ILCs.** Group 1 ILCs comprise conventional NK cells as well as interferon-γ producing ILCs. Group 2 ILCs produce Th2 like cytokines. Group 3 ILCs are further subdivided into 3 subsets, including LTi cells, RORγt^{+} ILCs that lack NK cell cytotoxicity receptors (NCR), such as NKp46, and RORγt^{+} ILCs that express NCRs. (Adapted from Spits et al., 2013).

Lymphoid Tissue inducer cells

Lymphoid Tissue inducer (LTi) cells are the prototypical RORγt$^+$ ILC lineage. They were first described in 1992 by Kelly and Scollay as CD3$^-$ CD8$^-$ CD4$^+$ IL7R$^+$ cells populating neonatal LNs, but then progressively decline with time as thymic T-cell production begins and lymphocyte migration to the LNs occurs (Kelly and Scollay, 1992). LTis account for 50% of the cells present in the LN at day 1 of life, declining to 1% by day 7 when CD3$^+$ CD4$^+$ T-cells begin to dominate (Kelly and Scollay, 1992). In the peripheral blood these cells peak during embryonic development (day 15) and steadily decline thereafter (Mebius *et al*., 1997). CD4$^+$ CD3$^-$ LTi cells express the gut homing receptor α4β7 that binds to its adhesion molecule target MAdCAM-1 that as well as being expressed in PPs, is also transiently expressed by the high endothelial venules of all LN for up to 24 hours after birth (Mebius *et al*., 1997). Secondary lymphoid organogenesis is initiated following interaction between lymphotoxin-β (LTβ) provided by LTis, and LTβ receptor expressed by stromal cells (Fu and Chaplin, 1999; Mebius *et al*., 1997).

LTi development is dependent on the transcription factor RORγt (Eberl *et al*., 2004). In humans, RORγt$^+$ IL7R$^+$ ILCs that lack other lineage markers, including CD4, are also found in the lymph nodes of the 8 week old foetus prior to the arrival of T-cells (Cupedo *et al*., 2009). Interest in ILCs as potential effector cells in autoimmune diseases, including IBD have been galvanised following studies showing that both human (Cupedo *et al*., 2009) and murine (Takatori *et al*., 2009) RORγt$^+$ LTi cells are a potent source of IL-17 and IL-22. Furthermore, conditional RORγt lineage tracing studies have shown that in adult mice the majority of RORγt$^+$ ILCs reside in the intestine (Sawa *et al*., 2010).

Intestinal RORγt$^+$ innate lymphoid cells

NK cells are found in the spleen, liver, other secondary lymphoid tissue and indeed circulating in peripheral blood. However, some NK cell populations occupying the small and large intestine appear to be distinct lineage. Conventional NK cells express the Th1 type transcription factors, such as T-bet and eomesodermin (Gordon *et al*., 2012; Townsend *et al*., 2004). They are a rich innate source of IFNγ, possess cytotoxic functions and are involved in host defence against viruses, neoplastic

change and cell senescence (Vivier *et al*., 2008). In the gut, cells expressing the NK marker NKp46 can be subdivided into 3 populations according to their expression of NK1.1 and IL-7R (CD127) (Satoh-Takayama *et al*., 2008). NK1.1^{+} CD127^{-} cells resembling conventional NK cells accounted for only about a third of small intestinal NKp46 expressing cells. Like conventional NK cells, these cells produced IFNγ and perforin. However, the most frequently observed NKp46^{+} population were NK1.1^{-} CD127^{+} cells, which lacked other NK markers, such as CD94, Ly49A/D and CD49b and did not produce IFNγ or perforin (Satoh-Takayama *et al*., 2008). NKp46^{+} NK1.1^{-} CD127^{+} cells also lacked NK receptors such as TRAIL and FasL (Satoh-Takayama *et al*., 2008), that are required to trigger apoptosis in target cells, indicating that these cells are not "natural killers" and instead have a functionally distinct role. Intestinal NKp46^{+} NK1.1^{-} CD127^{+} cells also expressed the transcription factor RORγt which is not found in conventional NK cells (Satoh-Takayama *et al*., 2008). Genome-wide expression profiling has shown that intestinal NKp46^{+} NK1.1^{+} CD127^{-} cells strongly resemble conventional splenic NK cells, whereas NKp46^{+} NK1.1^{-} CD127^{+} cells have a completely different expression profile and their transcriptome resembles that of RORγt^{+} LTi cells (Reynders *et al*., 2011). Furthermore, lineage tracing studies indicate that RORγt^{+} LTi like cells differentiate into NKp46^{+} RORγt^{+} intestinal ILCs, but do not give rise to conventional NK cells. For example, RORγt lineage tracing (RORγt-EGFP) can be used to track the fate of cells that express RORγt (Eberl *et al*., 2004). Adoptive transfer of RORγt^{+} LTi cells into *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice that lack endogenous NK cells and ILCs, resulted in differentiation of both NKp46^{+} RORγt^{+} and NKp46^{-} RORγt^{+} ILC populations. In contrast, adoptively transferred NK cells failed to differentiate into any RORγt^{+} populations, and instead remained NKp46^{+} RORγt^{-} even when followed for 6 months post transfer (Vonarbourg *et al*., 2010). These data strongly suggest that NKp46^{+} RORγt^{+} ILCs are indeed a distinct lineage from conventional NK cells.

Instead of producing IFNγ, NKp46^{+} RORγt^{+} ILCs are a rich source of IL-22 (Sanos *et al*., 2009; Satoh-Takayama *et al*., 2008), which is a key cytokine involved in early host immunity to intestinal pathogens, such as *Citrobacter rodentium* (Zheng *et al*., 2008). Induction of IL-22 peaks at just 4 days post inoculation of *C. rodentium* and is dependent on host expression of IL-23 (Sonnenberg *et al*., 2011; Zheng *et al*., 2008). Susceptibility to *C. rodentium* infection is increased in *Il22*$^{-/-}$ mice or following IL-

22 neutralisation (Sonnenberg *et al*., 2011; Zheng *et al*., 2008). IL-22 dependent resistance to infection was also observed in *Rag2*$^{-/-}$ mice, indicating that innate immune mechanisms likely accounted for early host immunity to *C. rodentium* (Zheng *et al*., 2008). Indeed, IL-22 expressing innate immune populations out number IL-22 producing T-cells during the first 8 days of *C. rodentium* infection (Sonnenberg *et al*., 2011). *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice that lack ILCs altogether are incapable of IL-22 production and rapidly succumb to *C. rodentium* infection (Satoh-Takayama *et al*., 2008). However, a recent report has suggested that the dominant innate source of IL-22 in *C. rodentium* infection is in fact CD3^{-} IL-7R^{+} RORγt^{+} ILCs that expressed CD4 but not NKp46 (Sonnenberg *et al*., 2011). *In vivo* depletion of CD4 expressing cells severely impaired *C. rodentium* resistance in *Rag1*$^{-/-}$ mice illustrating an important functional role for IL-22 expressing CD4^{+} ILCs in *C. rodentium* infection (Sonnenberg *et al*., 2011).

There are also populations of IL-22 producing NK like cells in human mucosal associated lymphoid tissue (MALT), including the tonsils, PPs and appendix (Cella *et al*., 2009). These cells expressed NKp44, CCR6 and produced IL-22 in response to stimulation with IL-23 and to a lesser extent IL-6. These cells lacked perforin, granzyme B and did not produce IFNγ, consistent with their lineage separation from conventional NK populations. It has also been reported that IL-22 producing NKp44^{+} IL7R^{+} RORγt^{+} cells are present in non-inflamed human gut and are reduced in CD patients (Takayama *et al*., 2010).

In addition to RORγt^{+} IL-22 producing ILCs that confer protective immunity to intestinal pathogens in mice, it is now recognised that an additional population of RORγt^{+} intestinal ILCs exist that mediate intestinal inflammation. Infection with the mouse pathogen *Helicobacter hepaticus* (*Hh*) induces typhlocolitis and liver disease in susceptible mice strains (Fox *et al*., 1994; Ward *et al*., 1996). Chronic colitis in *Hh* infected mice is IL-23 dependent and is fully penetrant in 129 SvEv *Rag1*$^{-/-}$ mice lacking adaptive immunity (Hue *et al*., 2006), highlighting the importance of innate immune mechanisms in this model of IBD. It has recently been shown that chronic colitis in *Hh* infected *Rag1*$^{-/-}$ mice is mediated by intestinal RORγt^{+} ILCs (Buonocore *et al*., 2010). These cells expressed hallmark ILC markers, including CD90, IL-7R, Sca-1, CD44 and CD25, lacked expression of NKp46, CD4 or other lineage markers,

and produced IL-17, IL-22 and IFNγ (Buonocore *et al*., 2010). Chronic colitis in *Hh* infected *Rag1*$^{-/-}$ mice is ameliorated by depleting CD90 expressing cells or by simultaneously blocking IL-17 and IFNγ (Buonocore *et al*., 2010). This group were also able to show that innate immune mediated colitis induced by administration of agonistic anti-CD40 mAbs to *Rag1*$^{-/-}$ mice was also driven by RORγt^{+} ILCs, and notably *Rag1*$^{-/-}$ *Rorc*$^{-/-}$ double knockout mice were resistant to disease (Buonocore *et al*., 2010). These results indicate that inflammatory ILCs also exist in the intestine and that in the appropriate context these cells are potent inducers of intestinal inflammation. RORγt^{+} ILCs were also seen in immunocompetent mice following induction of colitis with *Hh* (Buonocore *et al*., 2010), indicating that these cells are not merely an artefact of mice lacking adaptive immunity, but may indeed play a functionally relevant role in IBD. Recent data indicate that IL-1β, an inflammatory cytokine, which is upregulated in the intestinal LP of IBD patients (Reinecker *et al*., 1993), may also drive inflammatory ILC effector function (Coccia *et al*., 2012). IL-1β is elevated in ILC mediated models of colitis, such as *Hh* induced disease (Coccia *et al*., 2012). ILCs are the major intestinal cell population expressing the IL-1β receptor and IL-1β blockade significantly reduced ILC numbers and attenuated *Hh* induced colitis in lymphopaenic mice (Coccia *et al*., 2012).

The Powrie group has also shown that IL-23 responsive ILCs are present in inflamed gut of CD patients (Geremia *et al*., 2011). Transcripts encoding IL-17 and IL-22 were further enriched in the non T-cell fraction of cLPLs from both UC and CD patients in comparison with non-IBD controls and are further upregulated following IL-23 stimulation (Geremia *et al*., 2011). The innate population of IL-17 producing cells lacked major lineage markers (CD3, CD19, CD14, CD16, CD56) but did express the IL-7R, consistent with an ILC phenotype (Geremia *et al*., 2011). In contrast, the major producers of IL-22 were lin^{-} CD56^{+} cells (Geremia *et al*., 2011), consistent with the likelihood that these cells are the NK like mucosal cells observed by other groups that have been shown to co-express CD56 and NKp44 (Cella *et al*., 2009; Cupedo *et al*., 2009; Takayama *et al*., 2010).

GATA-3$^+$ innate lymphoid cells

ILCs that express Th2 cytokines have been termed nuocytes (Neill *et al*., 2010), innate helper 2 cells (Price *et al*., 2010), natural helper cells (Chang *et al*., 2011) and ILC2 cells (Spits and Cupedo, 2011). Like Th2 CD4$^+$ cells, these cells express the Th2 lineage defining transcription factor GATA-3 (Hoyler *et al*., 2012; Mjosberg *et al*., 2012) and produce IL-5 and IL-13, but not IFNγ or IL-22 (Hoyler *et al*., 2012). Like other ILC populations, GATA-3$^+$ ILCs are dependent on IL7R signalling and are increased in the absence of adaptive immunity (Hoyler *et al*., 2012). However, ILC2 development was independent of RORγt and AhR (Hoyler *et al*., 2012). In the first 2 months after birth GATA-3$^+$ ILCs express the gut homing integrin α4β7 and are found in 10-20 fold higher numbers in the small and large intestine in comparison with other mucosal tissues such as the lung (Hoyler *et al*., 2012). The migration of GATA-3$^+$ ILC2 cells to the intestine is CCR9 dependent (Hoyler *et al*., 2012). In mice, GATA-3$^+$ ILCs contribute to intestinal helminth expulsion in an IL-13 dependent manner (Hoyler *et al*., 2012). GATA-3$^+$ ILCs are present in human mucosal tissue (Mjosberg *et al*., 2012). These cells respond to typical "Th2" signals, such as IL-33 and TSLP to induce IL-4, IL-5 and IL-13. Effector function of human GATA-3$^+$ ILCs can be subdued by silencing GATA-3 with small, hairpin RNA sequences (Mjosberg *et al*., 2012), highlighting the importance of this transcription factor in the maintenance of effector function of these cells.

ILC development and maintenance

A population of IL-7R$^+$ Sca-1^{low} c-Kit^{low} progenitors found in the foetal liver are thought to give rise ILCs, since adoptive transfer of these progenitors to irradiated hosts gives rise to all lymphoid lineages, including T-cells, B-cells, NK cells and a population of CD45$^+$ CD4$^+$ CD3$^-$ ILCs (Mebius *et al*., 2001). These IL-7R$^+$ Sca-1^{low} c-Kit^{low} precursors resemble common lymphoid precursors that reside in the bone marrow, which seed all lymphoid lineages in adult mice (Mebius *et al*., 2001). The transcriptional and molecular control of ILC development has been scrutinized in recent years and various transcription factors have been identified which play an indispensable role in the development of mature ILCs, including RORC, RORα, Id2 (inhibitor of DNA binding 2), Ahr (aryl hydrocarbon receptor) and Tox (thymocyte selection associated high-mobility group box protein). Eberl *et al* generated

RORγt$^{GFP/GFP}$ reporter mice that have a fluorescent protein and stop codon inserted immediately downstream of the start codon of exon 1γt, which is the splice variant of the *Rorc* gene that generates the mature RORγt protein. The *RORγt*$^{GFP/+}$ heterozygote can be used to track the fate and tissue distribution of RORγt expressing cells and the homozygote (*RORγt*$^{GFP/GFP}$) is essentially *RORγt* null (Eberl *et al*., 2004). As well as confirming that CD45$^+$ CD3$^-$ CD4$^+$ IL-7R$^+$ LTis are one of the earliest cells found in embryonic LN and PP anlagens and that RORγt is exclusively expressed by ILCs in the mouse embryo, it was also shown that RORγt is indispensable for ILC generation and LN and PP formation (Eberl *et al*., 2004).

All ILC subsets described to date, including RORγt$^+$ and GATA-3$^+$ ILCs express the helix-loop-helix (HLH) transcription factor Id2 (Hoyler *et al*., 2012). *Id2*$^{-/-}$ mice have severely diminished numbers of IL-7R$^+$ CD3$^-$ CD4$^+$ LTi cells and also lack LNs and PPs. Id2 is a transcriptional inhibitor that forms heterodimers with another family of transcription factors called E proteins that play a key role in the development and differentiation of different immune cell lineages. Id2 binds and sequesters E2A that otherwise inhibits ILCs and NK cell development (Boos *et al*., 2007). ILCs can be restored and secondary lymphoid organogenesis rescued in *Id2*$^{-/-}$ *E2a*$^{-/-}$ double knockout mice, consistent with the notion that Id2 acts by functionally silencing E2A mediated suppression of NK and ILC ontogeny (Boos *et al*., 2007). Unlike *RORγt*$^{-/-}$ mice, *Id2*$^{-/-}$ mice also lack NK cells and some myeloid lineages, such as CD11b$^+$ cells, consistent with the requirement for Id2 in the development of other immune lineages (Yokota *et al*., 1999). Another transcription factor, Tox, has also been shown to be critical for the development of numerous lymphoid lineages, including CD4$^+$ T-cells, NK cells and LTis, however, some RORγt$^+$ NKp46$^+$ cells were reported to be present in the small intestine of *Tox*$^{-/-}$ mice, indicating that some ILC populations may develop independently of Tox (Aliahmad *et al*., 2012). There is impaired lymphoid organogenesis, including absent or severely hypoplastic LN and PPs in *Tox*$^{-/-}$ mice consistent with the marked reduction in LTi cells (Aliahmad *et al*., 2012).

Recently, GATA-3$^+$ ILCs have been shown to develop from Sca-1^{high} Id2$^+$ GATA-3^{high} precursors in the bone marrow (Hoyler *et al*., 2012), and also from common lymphoid progenitor cells (Wong *et al*., 2012), which are even earlier progenitors.

Notably, cytokines that promote the differentiation of other innate lymphoid lineages, such IL-7 and IL-15 (NK cells), or IL-1β, IL-2, IL-7 and IL-23 (RORγt^{+} ILCs) could not deviate the differentiation of Sca-1^{high} Id2^{+} GATA-3^{high} precursors into other ILC subsets indicating that these cells are committed precursors (Hoyler *et al*., 2012). GATA-3 is also required for maintenance of ILC2 cells, since conditional deletion of *Gata3* in Id2 expressing cells using the cre-lox recombinase system effectively depletes all mature IL-13 producing intestinal ILCs (Hoyler *et al*., 2012). ILC2 cells are also dependent on the transcription factor RORα, since staggerer mice (*Rora*$^{st/st}$) that have a spontaneous deletion of *RORa* are unable to generate intestinal ILC2 cells rendering them highly susceptible to helminth infection (Wong *et al*., 2012). Mouse and human ILCs express the aryl hydrocarbon receptor (Ahr) transcription factor (Cella *et al*., 2009; Takatori *et al*., 2009), an intracellular sensor that detects xenobiotics and endogenous ligands including tryptophan metabolites, flavinoids and arachidonic acid. Ahr is expressed by Th17 cells, and Th17 differentiation has been shown to be augmented by natural Ahr agonists and blocked by Ahr antagonists (Quintana *et al*., 2008), underlining the phenotypic similarities between some ILC populations and the CD4^{+} Th17 effector lineage. NKp46^{+} IL7R^{+} ILCs are absent and other ILC populations diminished in *Ahr*$^{-/-}$ and consequently these mice have impaired IL-23 induced IL-22 production and are highly susceptible to *C. rodentium* infection (Lee *et al*., 2012). Ahr may exert some of its biological activities in ILCs through the regulation of Notch signalling. Transcripts encoding *Notch1* and *Hes1* (a gene induced by Notch signalling) are upregulated in Ahr^{high} NKp46^{+} cells in comparison with Ahr^{low} cells. Furthermore, Notch1 and Notch2 could also be induced by Ahr agonists, or in cell lines where Ach was constitutively activated (Lee *et al*., 2012). Additional support for the role of Notch in ILCs was demonstrated by the reduction in ILCs observed in RBP-J$_{\kappa}$ mice that lack the transcription factor RBP-J$_{\kappa}$ which is the transcriptionally active component of Notch signalling (Lee *et al*., 2012).

Organised lymphoid tissue is not required for intestinal ILC development, since the formation of secondary lymphoid structures is dependent on LTβ provided by LTi cells interacting with the LTβ receptor expressed by mesenchymal cells. However,

IL-22 producing intestinal ILCs developed unhindered in mice lacking the LTβ receptor (Satoh-Takayama *et al*.).

Innate lymphoid cells and the intestinal microbiota

Although a role for ILCs is emerging in host resistance to intestinal infections, there are conflicting data regarding the role of the normal intestinal microbiota and mucosal ILCs. In one study, using RORγt lineage tracing mice, the number of intestinal RORγt$^+$ ILC populations was comparable in germ free, conventionally colonised and antibiotic treated mice, as well as in mice lacking the microbial sensing molecules Nod1, Nod2 or Myd88 (Sawa *et al*., 2010). These data have been corroborated in other studies (Reynders *et al*., 2011; Sonnenberg *et al*., 2012) and are in keeping with the identification of RORγt$^+$ ILCs in the intestine of germ-free embryo (Reynders *et al*., 2011). However, other groups have reported that the intestinal microbiota does impact ILC development and/or phenotype. For instance it has been reported that although conventional NK cells developed unhindered in germ free conditions, NKp46$^+$ NK1.1$^-$ IL-7R$^+$ ILC numbers were severely diminished in germ free mice (Satoh-Takayama *et al*., 2008). Evidence supporting impaired ILC development in the absence of gut commensals has also been provided by other groups (Sanos *et al*., 2009; Vonarbourg *et al*., 2010). It has also been suggested that IL-22 producing ILCs can influence the profile, distribution and containment of the intestinal microbiota (Sonnenberg *et al*., 2012). In this study depletion of ILCs resulted in systemic dissemination of microbes that could be rescued by replenishing IL-22 (Sonnenberg *et al*., 2012). It is intuitive that IL-22 producing ILCs are likely to play a role in intestinal barrier function since IL-22 induces the production of many antimicrobial peptides by intestinal epithelial cells, including RegIIIβ, RegIIIγ, haptoglobin, SAA3, S100A8, S100A9 and lactotransferrin (Zheng *et al*., 2008). The role of the microbiota in inducing and propagating ILCs is in need of further study.

Cytokine requirements for innate lymphoid cells

Valuable insights have been gained regarding the cytokine requirements for ILCs in various cytokine and cytokine receptor knockout mice. ILCs are virtually absent in *Rag2*$^{-/-}$ mice additionally lacking the common γ chain cytokine receptor (*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$), a key subunit for the signalling of numerous cytokines, including IL-2, IL-4,

IL-7, IL-9, IL-15 and IL-21 (Luci *et al*., 2009; Satoh-Takayama *et al*., 2008; Takatori *et al*., 2009). However, *Rag2*$^{-/-}$ *Il2rb*$^{-/-}$ mice lacking the IL2Rβ chain, which is required for IL-2 and IL-15 signalling, have increased numbers of ILCs, indicating that these cytokine are dispensable for ILC development (Satoh-Takayama *et al*., 2008). In keeping with these observations, NKp46^{+} NK1.1^{-} IL7R^{+} ILC ontogeny is unaffected in *Il15*$^{-/-}$ mice, whereas there is a complete failure of conventional NK cell development (Satoh-Takayama *et al*., 2008). Crucially, intestinal RORγt^{+} ILCs are significantly diminished in *Il7*$^{-/-}$ or *Il7r*$^{-/-}$ mice (Vonarbourg *et al*., 2010), indicating that among the common γ chain cytokine receptor family members IL-7 signalling plays a central role in ILC development and/or maintenance.

Cytokines such as IL-6, IL-12 and IL-23 appear to be less important for the development or maintenance of ILCs. In one study, using fate mapping with RORγt lineage tracing mice, there was a minor reduction in the number of RORγt^{+} ILCs in the small intestine of *Il6*$^{-/-}$ mice (Satoh-Takayama *et al*., 2008), whereas another study found only a negligible reduction in intestinal ILCs in *Il6*$^{-/-}$ mice (Sanos *et al*., 2009). RORγt^{+} ILCs develop normally in *Il12rb1*$^{-/-}$ mice unable to respond to IL-12 or IL-23 (Satoh-Takayama *et al*., 2008), indicating that these cytokines are dispensable for ILC development.

1.3.2.2 Intestinal dendritic cells

The term dendritic cell (DC) was first used to describe a population of adherent cells present in lymphoid tissues in 1973 (Steinman and Cohn, 1973). They were described as large cells of varying shape, including elongated bipolar cells and elaborate, stellate and dendritic cells with striking differences from macrophages (Steinman and Cohn, 1973). Ralph Steinman was awarded the Nobel Prize for Physiology in 2011 for these observations. DCs are specialised antigen presenting cells (APCs) that play a key role orchestrating the immune response. DCs initially occupy non-lymphoid tissues of the body, such as the intestinal lamina propria in an immature state surveying antigens before migrating from their native location to secondary lymphoid structures, typically draining LNs, where they then present antigen (Ag) to CD4^{+} T-cells (Steinman, 1991). In keeping with their role as sentinels intestinal DCs have been observed to project their dendrites between intestinal epithelial cells and directly sample fluorescent labelled bacteria present in

the gut lumen (Chieppa *et al*., 2006; Rescigno *et al*., 2001). DCs express proteins involved in tight junction formation, such as claudin-1, occludin, β-catenin and E-cadherin enabling them to form close contacts with epithelial cells and penetrate their dendrites between epithelial cell tight junctions without losing the integrity of the brush border barrier (Rescigno *et al*., 2001). Intra-vital studies using mice engineered to express fluorescent proteins under control of either the CD11c or MHC class II promoters (classical DC markers) have elegantly visualised DCs directly sampling luminal bacteria *in vivo* in real time (Chieppa *et al*., 2006). Transepithelial dendrite protrusions are enhanced by exposing the epithelium to microbes or their products (Chieppa *et al*., 2006; Rescigno *et al*., 2001) and can be decreased by reducing the microbial load in the lumen with antibiotics (Chieppa *et al*., 2006), indicating that the intestinal microbiota influence this process directly. Bone marrow chimaeras using *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$ and *Myd88*$^{-/-}$ hosts reconstituted with either CD11c-EGFP or MHCII-EGFP bone marrow cells convincingly demonstrated that DC epithelial extension were responsive to Toll-like receptor (TLR) agonists and were dependent on signalling through the TLR adaptor molecule MyD88 (Chieppa *et al*., 2006).

In the steady state, tissue dwelling DC, such as those occupying the cLP, express low levels of co-stimulatory molecules and instead these cells are predominantly pinocytic, in keeping with their role sampling antigens in their immediate surroundings (Cruickshank *et al*., 2005; Krajina *et al*., 2003). However, once DCs have captured and processed Ag they migrate to LNs where they present Ag-derived peptides in the context of MHC to CD4^{+} T-cells (Banchereau and Steinman, 1998). DCs express surface co-stimulatory molecules, such as CD40, CD80, CD83 and CD86 that help to prime T-cell activation (Banchereau and Steinman, 1998). During the presentation of Ag, DCs produce cytokines that play a key role shaping the differentiation of particular functional T-cell lineages, including IL-12 which is required for Th1 differentiation (Macatonia *et al*., 1995) and IL-23 which is required for the differentiation and/or maintenance of Th17 cells (Aggarwal *et al*., 2003).

DCs are distributed throughout the small and large intestine lamina propria, PPs, isolated lymphoid follicles (ILFs) and mLN, and some of these lymphoid tissues are key inductive sites of T-cell activation (Rescigno and Di Sabatino, 2009). Intestinal DCs have been subdivided in different subsets that are phenotypically and

functionally distinct. Colonic $CD11c^{high}$ DCs can be broadly divided into 2 main subtypes comprising $CD103^+$ $CD11b^-$ and $CD103^-$ $CD11b^+$ cells (Denning *et al*., 2007). *Cx3cr1*-GFP lineage tracing mice have been used to demonstrate that CD11b expressing DCs can be further subdivided into 3 separate groups based on their expression of the chemokine receptor CX_3CR1 (Varol *et al*., 2009). This important study also provided crucial insights into the origins of the different intestinal lamina propria DC subsets. CD11c-DTR mice have the human diphtheria toxin receptor (DTR) cloned upstream of the CD11c promoter, which results in selective expression of the DTR in cell lineages that express CD11c, of which DCs are the prototypical example. When diphtheria toxin is administered to these mice it is selectively taken up by DTR^+ $CD11c^+$ DCs resulting in their selective depletion *in vivo*. By using this system and then reconstituting DC depleted mice with specific cellular precursors it was possible to identify which precursors were responsible for seeding the different intestinal DC populations. This study demonstrated that the majority of $CD11b^+$ $CX3CR1^+$ DCs are derived from circulating $Ly6C^{high}$ monocytes in a GM-CSF dependent manner. $CD103^+$ $CD11b^-$ DCs were derived from macrophage-dendritic cell precursors (MDPs) in a Flt3L dependent manner (Varol *et al*., 2009). This study also indicated that $CD103^+$ and $CD11b^+$ intestinal DCs are functionally distinct, particularly in the context of intestinal inflammation. Reconstitution of DC depleted mice with monocytes that are responsible for seeding $CD11b^+$ CX_3CR1^{high} precursors were found to have increased susceptibility to DSS induced colitis (Varol *et al*., 2009). However, reconstitution with $tnfa^{-/-}$ monocytes was only associated with mild disease, indicating that the $CD11b^+$ CX_3CR1^{high} DCs that they seed possess colitogenic properties that is at least partially dependent on the pro-inflammatory cytokine TNFα. $CD11b^+$ CX_3CR1^+ DCs have also been shown to potently promote the differentiation of Th17 cells (Denning *et al*., 2007).

It should also be remembered that DCs are part of a larger family of myeloid cells that together with monocytes and macrophages comprise the mononuclear phagocyte system. There is considerable overlap in the expression of some cell surface markers common to these different cell types, including CX_3CR1, and accordingly the depletion/re-engraftment type experiments described above should be interpreted in this light. Indeed, CX_3CR1 is broadly expressed by other cells of the mononuclear phagocyte system, including DCs (Varol *et al*., 2009), monocytes (Geissman *et al*.,

2003) and macrophages (Yona *et al*., 2013). Other myeloid markers are also shared between these related cells. For instance, F4/80, which is often considered a macrophage marker, is also expressed by some DCs (Hume 2008). Likewise, CD11c often considered a "pure" DC marker, is also expressed by macrophages (Hume 2008). Therefore, definitions of DCs or macrophages by particular cell surface markers alone can be confusing or even misleading in some experimental systems.

In contrast to $CD11b^+$ CX_3CR1^{high} DCs there is evidence linking $CD103^+$ DCs to the promotion of intestinal $FoxP3^+$ T-reg differentiation and the imprinting of gut homing properties to circulating lymphocytes. Naive $CD4^+$ T-cells differentiate into $Foxp3^+$ T-regs in the gut following oral administration of their antigen (Coombes *et al*., 2007), a process that has long been recognised to promote immunological tolerance. Ag-specific $FoxP3^+$ T-reg differentiation from naive precursor cells was increased 30-40 fold if these cells were primed with $CD103^+$ mLN DCs rather than $CD103^-$ DCs, in a TGF-β dependent manner (Coombes *et al*., 2007). Conventional $CD4^+$ $CD25^+$ $FoxP3^+$ T-regs isolated directly from the spleen could also be supported and sustained far more effectively by $CD103^+$ DCs (Coombes *et al*., 2007). The production of inflammatory cytokines, such as TNFα, IL-6, IL-12p40 and IL-23p19 was also diminished in $CD103^+$ DCs (Coombes *et al*., 2007). $CD103^+$ DCs, but not $CD103^-$ DCs from the mLN potently induced the gut homing molecules α4β7 and CCR9 in responder T-cells that they prime with antigen (Johansson-Lindbom *et al*., 2005). A similar observation was made in DCs isolated from human mLNs, with enhanced induction of CCR9 observed in responding T-cells interacting with $CD103^+$ DCs, however, α4β7 induction on T-cells was comparable with $CD103^+$ and $CD103^-$ DCs (Jaensson *et al*., 2008).

DCs have been implicated in the pathogenesis of IBD. Recently an attempt has been made to try to link disease susceptibility genes identified in a meta-analysis of the major GWA studies in IBD to specific cells types, and interestingly the greatest number of hits was found in DCs (Jostins *et al*., 2012). In experimental colitis there are increased numbers of DCs in the cLP and mLN, and there is marked upregulation of co-stimulatory molecules and pro-inflammatory cytokines by these cells (Cruickshank *et al*., 2005; Krajina *et al*., 2003). Activation of DCs and other myeloid cells with agonistic anti-CD40 antibodies has been shown to trigger colitis in mice

lacking T and B cells (Uhlig *et al*., 2006). Depletion of DCs in DSS colitis using CD11c-DTR mice also protected from disease in both WT and *Rag1*$^{-/-}$ mice indicating that this property was independent of T-cells (Abe *et al*., 2007). Studies in human IBD have also shown increased activation of intestinal DCs in inflamed mucosa. In contrast to non-inflammatory controls or non-inflamed intestinal segments from the same patient, lamina propria DCs from inflamed gut segments have increased expression of activation markers, such as CD40, and increased expression of pro-inflammatory cytokines, such as IL-12 and IL-6, which in the case of IL-6 also positively correlated with disease activity (Hart *et al*., 2005; Ng *et al*., 2011). DCs also express pattern recognition receptors, such as TLRs and in IBD patients there is significantly increased expression of TLR2 and TLR4 in lamina propria DCs (Hart *et al*., 2005). Recently, insights into how IBD susceptibility genes identified in GWA studies might impact on DC function in a disease relevant manner have been provided by analysing the phenotype of DCs from patients with polymorphisms in *NOD2*. MDP, the bacterial derived ligand for NOD2, triggers autophagosome formation and upregulation of autophagy in DCs in a NOD2 dependent manner (Cooney *et al*., 2010). MDP induced DC autophagy was dependent on recognised autophagy components, including ATG5, ATG7 and ATG16L1 and resulted in increased expression of MHC class II on the surface of DCs, linking NOD2 dependent autophagy to Ag presentation in DCs (Cooney *et al*., 2010). Crucially, DCs from individuals harbouring NOD2 variants associated with CD had markedly impaired MDP induced autophagy and failed to upregulate MHC class II (Cooney *et al*., 2010), consistent with the likelihood that disease associated NOD2 polymorphisms are linked to loss of autophagy/Ag presentation function in DCs.

1.4 Transcriptional regulation of the mucosal immune system

Mucosal immune responses are tightly regulated since the host must rapidly deploy aggressive, inflammatory responses in the event of pathogen exposure, yet at the same time exercise restraint against non-pathogenic agents by utilising regulatory mechanisms. The inflammatory and regulatory properties of mucosal immune cells are governed by cell-specific transcriptional programmes, which in turn are controlled by transcription factors that dictate which immune response genes are switched on or off at any given moment. Among the most important transcription factors involved in controlling T-cell effector function are T-bet (Szabo *et al*., 2000), RORγt (Ivanov *et al*., 2006), GATA-3 (Zheng and Flavell, 1997) and FoxP3 (Hori *et al*., 2003). These transcription factors are considered the master regulators responsible for controlling the fate and phenotype of Th1, Th17, Th2 and T-reg $CD4^+$ T-cells, respectively. Other notable transcription factors involved in the regulation of host immunity at the barrier surfaces include the signal transducer and activator of transcription (STAT) family of proteins and NFκB. The STATs, including STAT1, STAT3, STAT4 and STAT6 have important role in T-cell differentiation, but are also critically involved in innate immune cells, including macrophages, NK cells and DCs (Stark and Darnell, 2012).

1.4.1 T-bet

T-bet (T-box expressed in T-cells) is a 62kDa 530 amino acid protein encoded by the *Tbx21* gene on chromosome 11 in mice (Szabo *et al*., 2000). When it was first cloned in 2000 it was shown to be present in Th1 cells where it bound at the promoters of the *Il2* and *Ifng* loci. It was rapidly induced in naive T-cells within 24 hours under Th1 polarising conditions, but not under Th2 conditions (Szabo *et al*., 2000). T-bet was a potent transactivator of the *Ifng* gene and retroviral transduction of T-bet into primary $CD4^+$ T-cell upregulated IFNγ production (Szabo *et al*., 2000). T-bet even redirected committed effector Th2 cells towards the Th1 lineage. Retroviral transduction of T-bet into *in vitro* differentiated Th2 cells resulted in suppression of IL-4 and IL-5 and the emergence of IFNγ production (Szabo *et al*., 2000). These experiments identified T-bet as the key transcription factor responsible for directing the transcriptional control of Th1 cells.

T-bet plays a critical role in regulating systemic, mucosal and organ specific immune responses, such that changes in T-bet expression profoundly influence the balance between the development of autoimmunity or susceptibility to infection (Table 3). T-bet deficient mice are resistant to experimental models of autoimmunity, such as diabetes (Esensten *et al*., 2009), EAE (Bettelli *et al*., 2004), lupus (Peng *et al*., 2002) and colitis (Neurath *et al*., 2002), yet on the other hand have heightened susceptibility to infections including *Mycobacteria* (Sullivan *et al*., 2005), *Salmonella* (Ravindran *et al*., 2005), *Leishmania* (Cobb *et al*., 2009), *trypanosome* (Rosas *et al*., 2006) and viruses (Matsui *et al*., 2005; Svensson *et al*., 2005). Accordingly, T-bet must be rigorously regulated in a dynamic fashion to maintain the capacity to resist infection, without exposing the host to increased risk of autoimmunity, particularly at mucosal surfaces often in contact with commensal and pathogenic microorganisms.

Table 3. T-bet impacts disease phenotype of multiple inflammatory, infectious and neoplastic diseases of the mucosae. References cited in the table are linked to the original paper (Powell *et al*., 2010).

Disease	Changes in T-bet	Experimental/clinical observation	Host
Asthma	Absent[97]	Spontaneous asthma	Mouse
	Reduced[97,104]	Reduced expression in T cells from airways and peripheral blood in patients with asthma	Human
	Genetic polymorphism[105–107]	Increased risk of asthma and predictor of response to GC therapy	Human
Pulmonary TB	Absent[6]	Increased susceptibility	Mouse
IBD	Absent[5]	Resistance to Th1 models of colitis and heightened susceptibility to Th2 model of colitis	Mouse
	Absent in innate immune cells[63]	Spontaneous TNFα-dependent colitis	Mouse
	Over-expression[5]	Heightened susceptibility to Th1 model of colitis	Mouse
	Increased[5,51,52]	Elevated T-bet expression in gut lamina propria T cells from patients with Crohn's disease	Human
Celiac disease	Increased[53,58]	Elevated T-bet expression in duodenal biopsies and peripheral blood T cells, monocytes and B cells in patients with celiac disease	Human
Periodontitis	Increased[82,83]	Increased T-bet expression in lesions from patients with active disease	Human
Oral GvHD	Increased[90]	Increased T-bet expression in $CD8^+$ T cells in oral lesions	Human
Helicobacter infection	Absent[119]	Resistance to ***Helicobacter felis*** induced gastritis and gastric adenocarcinoma	Mouse
Colorectal cancer	Increased[112]	Increased expression of T-bet in CRC specimens was associated with a favorable prognosis	Human
	Absent in innate immune cells[64]	Drives colitis-associated CRC	Mouse
Genital HSV infection	Absent[11]	Increased susceptibility to infection	Mouse
	Genetic polymorphism[123]	Increased susceptibility	Human
HPV infection and cervical neoplasia	Increased[113]	Increased T-bet expression in tumor-infiltrating T-cells was associated with a favorable prognosis	Human

T-bet expression is almost exclusively restricted to the immune system, and to date its expression has been detected in $CD4^+$ T-cells (Szabo *et al*., 2000), $CD8^+$ T-cells (Szabo *et al*., 2000), B-cells (Szabo *et al*., 2000), monocytes (Lighvani *et al*., 2001), DCs (Lighvani *et al*., 2001; Lugo-Villarino *et al*., 2003), NK cells (Szabo *et al*., 2000) and ILCs (Buonocore *et al*., 2010). Although most work so far has concentrated on the role of T-bet in T-cells, its role in the innate immune system, as well as in coordinating the complex interplay between innate and adaptive immune components, may yet prove to be even more significant. The multi-layered effects of T-bet on different cell types, particularly in the mucosal surfaces, are depicted in Figure 3.

Transcriptional targets of T-bet include cytokines, cytokine receptors, other transcription factors, and genes encoding proteins involved in cell metabolism, trafficking and cellular differentiation (Jenner *et al*., 2009). Like other immune transcription factors, such as STAT4 and STAT6 (Wei *et al*., 2010), T-bet tunes epigenetic modifications at loci encoding key immune proteins. It interacts with histone modifying enzymes such as H3K9 methyltransferase and H3K27 demethylase to alter the transcriptional activity of target loci, such as *Ifng* and *Cxcr3* (Chang and Aune, 2007; Fields *et al*., 2002; Lewis *et al*., 2007; Miller *et al*., 2008).

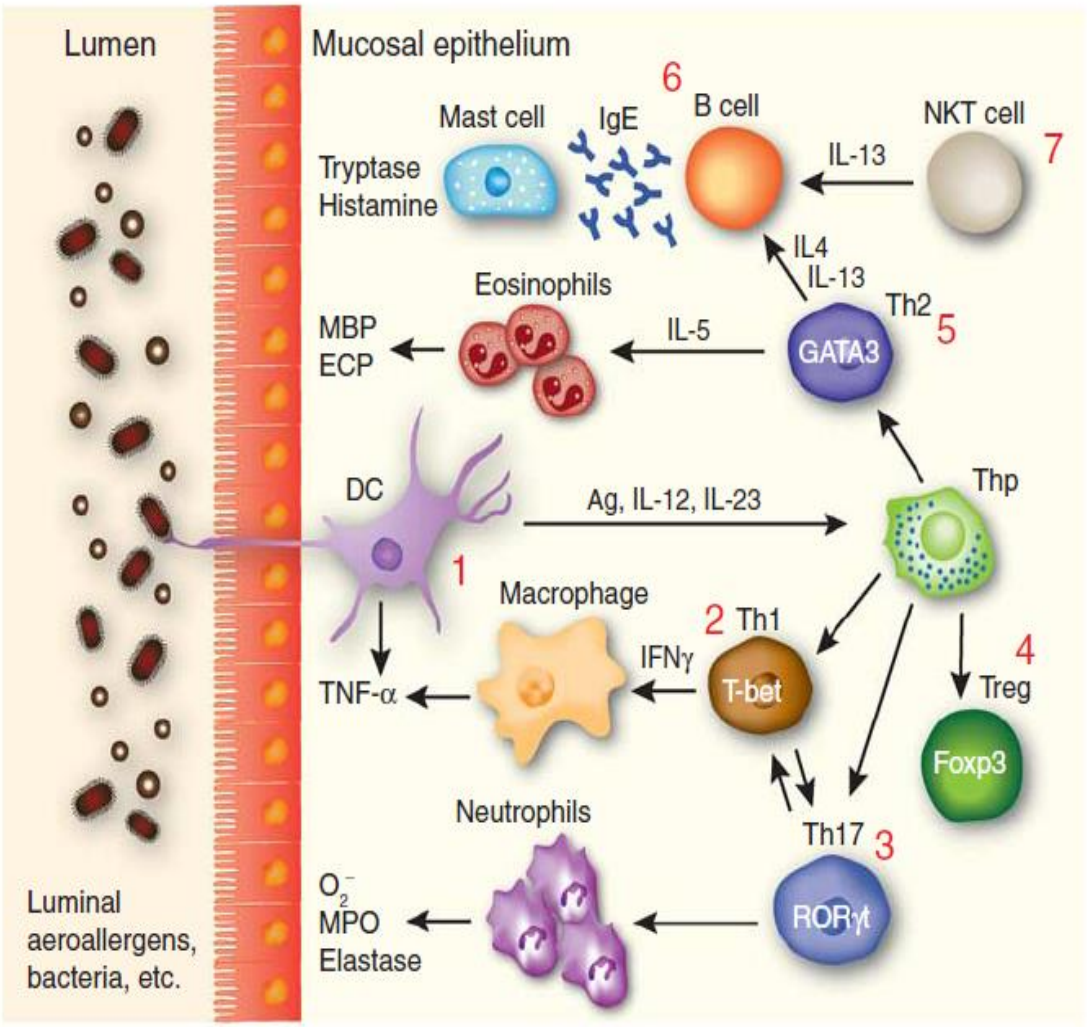

Figure 3. **T-bet mediated regulation of mucosal immunity.** 1 Loss of T-bet in DCs results in over-expression of TNFα, resulting in colonic epithelial apoptosis and colitis. T-bet expression in DCs is also required to recruit mast cells to gut. 2 Th1 cells provide resistance to intracellular organisms (e.g. mycobacteria)and are T-bet dependent. 3 In the absence of T-bet, Th17 cells preferentially expand. Th17 cells trigger neutrophil accumulation and are implicated in immunity to extracellular bacteria and IBD pathogenesis. 4 T-bet is implicated in Treg development (responsible for limiting Th1 responses). 5 In the absence of T-bet, Th2 cells expand and produce IL-4, IL-13, (Ig class switching to IgE and activation of mast cells), and IL-5 (expansion and activation of eosinophils). Eosinophilic inflammation is implicated in immunity to helminths and in the pathogenesis of asthma/rhinitis. 6 T-bet is inducible in B cells, leading to impairment of IgE class switching. Cross linking of IgE on mast cells activates mast cells. 7 Absence of T-bet leads to overexpression of IL-13 by mucosal NKT cells. Ag, antigen; ECP, eosinophil cationic protein; IFN, interferon; MBP, major basic protein; MPO, myeloperoxidase. Adapted from Powell *et al*., 2010.

1.4.1.1 The role of T-bet in $CD4^+$ T-cells

The mechanisms whereby T-bet directs Th1 polarisation are multifactorial and include direct transactivation of Th1 promoting genes, interaction with other transcription factors, epigenetic modification and repression of Th2 and Th17 differentiation. In Th1 cells alone T-bet binds at the promoter of more than 800 protein encoding genes (Jenner *et al*., 2009). T-bet transactivates the *Ifng* gene through binding to multiple proximal and distal cis-acting enhancer elements (Cho *et al*., 2003; Lee *et al*., 2004). Transactivation of other Th1 related genes, such *Tnfa* and *Il18rap* ensures that a coordinated and concerted Th1 response ensues (Jenner *et al*., 2009). Interactions between T-bet and other transcription factors may also contribute to Th1 lineage specification. T-bet interacts with STAT4 at multiple *Ifng* distal enhancer sites and recruits RelA to the *Ifng* locus to facilitate efficient transcription of the *Ifng* gene (Balasubramani *et al*., 2010).

T-bet also impacts on the differentiation and effector function of Th2, Th17 and T-reg lineages in functionally meaningful ways at the mucosal barrier. Th2 T-cells produce cytokines such as IL-4, IL-5 and IL-13 and play an important role driving allergic inflammatory disease, such as asthma and hay fever (Corrigan and Kay, 1992; Kay, 1991). T-bet mediated negative regulation of GATA-3 has also been proposed as a potential mechanism responsible for T-bet instigated Th1 differentiation and limitation of Th2 differentiation (Usui *et al*., 2006). Consistent with this notion, GATA-3 levels are increased in *Tbx21*$^{-/-}$ T-cells and forced expression of T-bet in Th2 cells suppresses GATA-3 expression (Usui *et al*., 2006). Although, GATA-3 is an important transcriptional regulator of Th2 differentiation, the interaction between T-bet and GATA-3 is a key event in determining the fate of T-cell lineage commitment. T-bet physically interacts with GATA-3, preventing it from binding to its transcriptional targets and repressing Th2 differentiation (Hwang *et al*., 2005). In addition to direct physical contact between these transcription factors, T-bet and GATA-3 also bind to a shared profile of target genes that are differentially expressed in Th1 and Th2 cells (Jenner *et al*., 2009). Accordingly, T-bet and GATA-3 interactions influence allergic inflammatory responses at mucosal surfaces. Notably, mice lacking T-bet spontaneously develop airway changes resembling human asthma with an eosinophil and T-cell rich inflammatory infiltrate,

overexpression of Th2 cytokines, increased peribronchial collagen deposition suggestive of airway remodelling, and bronchial hyper-responsiveness, (Finotto *et al*., 2002). Restoration of T-bet in T-cells reverses these changes (Park *et al*., 2009). T-bet expression is reduced in T-cells from the airways (Finotto *et al*., 2002) and blood (Ko *et al*., 2007) of patients with asthma. Polymorphisms at the *TBX21* locus are associated with an increased risk of asthma and airway hyper-responsiveness (Munthe-Kaas *et al*., 2008; Raby *et al*., 2006) and influence the response to inhaled corticosteroid therapy, the mainstay of asthma treatment (Tantisira *et al*., 2004).

In addition to their impaired Th1 differentiation, *Tbx21*$^{-/-}$ T-cells preferentially differentiate into Th17 cells (Durrant *et al*., 2009; Harrington *et al*., 2005; Park *et al*., 2005). T-bet inhibits Th17 development by inhibiting IL-23R expression (Harrington *et al*., 2005) and physically limiting another transcription factor, Runx1, from trans-activating the Th17 master transcription factor RORγt (Lazarevic *et al*., 2011). A complex interplay between T-bet and Th17 cells exists in disease models. Even though spontaneous asthma in *Tbx21*$^{-/-}$ mice is considered a Th2-mediated disease, adoptively transferred *Tbx21*$^{-/-}$ CD4^{+} cells also preferentially convert to Th17 cells, highlighting the importance of T-bet in limiting Th17 ontogeny (Fujiwara *et al*., 2007). Antigen-specific T-bet deficient T-cells preferentially differentiate into Th17 cells instead of Th1 cells and induce severe autoimmune disease (Villarino *et al*., 2010). However, in EAE T-bet appears to be required for disease not only in Th1 cells, but also in encephalitogenic Th17 cells (Yang *et al*., 2009). It is not certain whether T-bet confers pathogenic properties to Th17 cells, or whether these *in vivo* observations speak to issues regarding T-cell plasticity, since Th17 cells have been shown to differentiate into Th1 cells *in vivo* (Bending *et al*., 2009; Lee *et al*., 2009). One possibility is that T-bet is required for Th17 cells to differentiate into pathogenic Th1 cells.

T-bet is also upregulated in regulatory T cells (Tregs) in models of inflammation and infection, and its expression was required to suppress inflammation (Koch *et al*., 2009). Similar to Th1 cells, T-bet in Tregs was induced by Th1 conditions and required intact STAT1 and IFN-γR. One of the proposed mechanisms whereby T-bet is required for Tregs to exert control of inflammatory reactions is through transcriptional control of *CXCR3*. This chemokine receptor is a transcriptional target

of T-bet in both mice (Lord *et al*., 2005) and humans (Jenner *et al*., 2009), and its expression may facilitate Treg migration to sites of inflammation.

1.4.1.2 T-bet is expressed by cells of the innate immune system

T-bet expression has been detected in several innate immune cell types, including monocytes (Lighvani *et al*., 2001), DCs (Lighvani *et al*., 2001; Lugo-Villarino *et al*., 2003), NK cells (Soderquest *et al*., 2011; Szabo *et al*., 2000; Townsend *et al*., 2004) and ILCs (Buonocore *et al*., 2010).

1.4.1.3 The role of T-bet in NK cells

When T-bet was first identified it was observed that in addition to being expressed in Th1 cells, T-bet was also expressed by the NK cell line YT cells (Szabo *et al*., 2000). Subsequent work has shown that T-bet plays an important role in the homeostasis and maturation of NK cells. In *Tbx21*$^{-/-}$ mice there are significantly fewer NK cells in the spleen, liver and peripheral blood (Townsend *et al*., 2004). In addition to their numerical deficit, NK cells in T-bet deficient hosts exhibit maturation delay and evidence of hyperactivation. Recent data from our laboratory has shown that unlike WT NK cells that predominantly differentiate into a CD27^{lo} CD11b^{high} population of mature NK cells, in T-bet deficient hosts NK cell maturation is arrested at the CD27^{high} CD11b^{high} stage (Soderquest *et al*., 2011). This defect was previously shown to be specific to the haemopoeitic system, since reconstitution of sub-lethally irradiated WT mice reconstituted with *Tbx21*$^{-/-}$ bone marrow cells also exhibited defective terminal maturation of NK cells, whereas reconstitution of irradiated *Tbx21*$^{-/-}$ mice with WT bone marrow cells rescued this maturation arrest (Townsend *et al*., 2004). This T-bet dependent maturation defect is dependent on monocytes, which are able to facilitate the transition of CD27^{high} CD11b^{high} precursors in to mature CD27^{lo} CD11^{high} NK cells (Soderquest *et al*., 2011). T-bet expression is induced in NK cells by IL-12 and IL-15. Although by themselves, IFNγ and IL-18 do not induce significant T-bet expression in NK cells, both of these cytokines potentiate IL-12 induced T-bet expression (Townsend *et al*., 2004). T-bet deficient NK cells also appear to have defective effector function with reduced killing of YAC-1 target cells (Townsend *et al*., 2004). *Tbx21*$^{-/-}$ mice have markedly reduced absolute numbers of invariant NK T-cells (Townsend *et al*., 2004). Like *Tbx21*$^{-/-}$ CD4^{+} T-cells these cells have defective interferon-γ production and instead overproduce Th2 cytokines (Kim *et al*., 2009).

1.4.1.4 The role of T-bet in dendritic cells

T-bet is inducible in myeloid populations, including DCs, monocytes and monocytic cell lines by exposure to IFNγ (Lighvani *et al*., 2001; Lugo-Villarino *et al*., 2003). T-bet protein expression has also been demonstrated in DCs ($S\text{-}100^+$ cells) in human colon in the steady state, indicating a potential role for this transcription factor in gut DCs (Garrett *et al*., 2007). The functional role of T-bet in DCs has been addressed in animal studies utilising T-bet deficient mice. In one study it was shown that immunomagnetically sorted $CD11c^+$ DCs from *Tbx21*$^{-/-}$ mice were poor inducers of Th1 effector responses in comparison with WT DCs (Lugo-Villarino *et al*., 2003). Conversely, primary $CD11c^+$ DCs transduced with T-bet were more effective at promoting Th1 responses and cytotoxic $CD8^+$ T-cell responses and were able to slow tumour growth (Qu *et al*., 2010), indicating that T-bet is required for DCs to initiate optimal inflammatory responses. Similarly, *Tbx21*$^{-/-}$ mice which are resistant to autoimmune arthritis have fully restored disease susceptibility when T-bet sufficient DCs are adoptively transferred (Wang *et al*., 2006). In this study T-bet deficient DCs were inferior at priming specific antigen-specific T-cell proliferation and effector cytokine responses (both IFNγ and IL-4). T-bet expression in DCs also influences T-cell polarization in the airways. Sensitized mice receiving antigen pulsed DCs intranasally are unable to redirect pro-allergic Th2 airways responses towards Th1 responses in the absence of T-bet (Heckman *et al*., 2008).

However, mice lacking T-bet in the innate immune compartment (*Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$) develop spontaneous colitis, which is characterised by increased expression of TNFα (Garrett *et al*., 2007). The central role played by DCs in TRUC disease was demonstrated by reconstituting lethally irradiated TRUC mice with CD11c DTR tg DCs. Following engraftment of CD11c DTR DCs in the colon of TRUC mice diphtheria toxin was administered to chimeric mouse groups (TRUC grafted with TRUC bone marrow and TRUC grafted with CD11c DTR tg bone marrow). Diphtheria toxin mediated deletion of colonic DCs attenuated disease in TRUC mice, highlighting a key role of DCs in this model of IBD (Garrett *et al*., 2009). One of the key transcriptional targets of T-bet in DCs is the gene encoding the inflammatory cytokine TNFα. There are T-bet consensus sites upstream from the transcriptional start of the *Tnfa* locus (Garrett *et al*., 2007), and chromatin immunoprecipitation (ChiP) studies confirm that T-bet binds at the TNFα promoter (Garrett *et al*., 2007).

In contrast to its role in T-cells, T-bet appears to be a transcriptional repressor of TNFα in myeloid lineages. Transfection of T-bet into the RAW cell line results in suppression of *Tnfa* promoter activity in a luciferase assay (Garrett *et al*., 2007). These data are consistent with the observed overproduction of TNFα in DCs genetically deficient of T-bet. Further evidence that T-bet was a transcriptional repressor of the *Tnfa* locus in DCs was shown by generating TRUC mice that over-expressed T-bet under control of the CD11c promoter. Ectopic over-expression of T-bet in DCs resulted in repression of TNFα and reduced colitis (Garrett *et al*., 2009).

T-bet expression in DCs also impacts other innate lineages and has been implicated in the homeostatic homing of mast cells to the small intestine (Alcaide *et al*., 2007). *Tbx21*$^{-/-}$ mice have reduced numbers of intestinal mast cells and their progenitors. Mast cells from T-bet null hosts have diminished binding to VCAM-1 and MAdCAM-1, whose expression on small intestinal vascular endothelium is responsible for mast cell recruitment. Although T-bet expression has not been detected in mast cells at any stage during ontogeny, adoptive transfer of T-bet sufficient DCs restores normal mast cell homing to the small intestine, implying that T-bet indirectly influences mast cell trafficking to the gut through its actions in DCs.

1.4.1.5 T-bet and intestinal inflammation

T-bet has been shown to play an important role in preclinical models of gastrointestinal inflammation that are reliant on Th1 T-cells. Experimental colitis secondary to adoptive transfer of naive T-cells, TNBS administration and the spontaneous colitis developing in *Il10*$^{-/-}$ mice, are all associated with increased expression of T-bet in lamina propria T-cells (Neurath *et al*., 2002). In the naive T-cell transfer model of colitis, recipients of *Tbx21*$^{-/-}$ T-cells do not develop disease, whereas recipients of T-cells overexpressing T-bet develop accelerated disease (Neurath *et al*., 2002). In contrast, Th2 mediated murine models of inflammatory bowel disease (IBD), such as oxazolone induced colitis and the TCR-$\alpha^{-/-}\mu^{-/-}$ spontaneous colitis model are not associated with increased T-bet expression in lamina propria T-cells and *Tbx21*$^{-/-}$ mice develop more severe oxazolone disease in association with increased expression of Th2 cytokines (Neurath *et al*., 2002).

In humans, elevated expression of T-bet has also been confirmed in lamina propria T-cells from patients with intestinal inflammation, most notably in Crohn's disease

(Matsuoka *et al*., 2004; Monteleone *et al*., 2005; Neurath *et al*., 2002) and coeliac disease (Monteleone *et al*., 2004), where Th1 cells have been implicated as effector cells (Fuss *et al*., 1996; Nilsen *et al*., 1998; Nilsen *et al*., 1995; Parronchi *et al*., 1997). Increased expression of T-bet has also been observed in peripheral blood T-cells, B-cells and monocytes of untreated patients with coeliac disease (Frisullo *et al*., 2009). Following implementation of gluten avoidance and disease remission, T-bet levels fell, implying that dynamic changes in T-bet expression may be a useful biomarker to track disease activity in coeliac disease.

In Crohn's disease T-cell stimulation through the T-cell receptor with anti-CD3 antibodies induces T-bet expression in lamina propria T-cells (Matsuoka *et al*., 2004; Monteleone *et al*., 2005). In the case of coeliac disease, where the offending antigen is known, stimulation of duodenal T-cells with specific peptide antigen also induces T-bet expression (Fina *et al*., 2008). In these human Th1 associated diseases, blockade of IL-21 (Fina *et al*., 2008; Monteleone *et al*., 2005), or interferon-γ (Monteleone *et al*., 2004) reduces the expression of T-bet in T-cells, whilst antagonism of the anti-inflammatory cytokine TGF-β induces T-bet expression (Di Sabatino *et al*., 2008), suggesting that other inflammatory and regulatory pathways converge with T-bet mediated pathways to influence the prevailing inflammatory response. Anti-inflammatory therapies, such as steroids, which are effective in the management of severe exacerbations of IBD, also inhibit T-bet expression in T-cells (Liberman *et al*., 2007). The activated glucocorticoid receptor physically interacts with T-bet in the nucleus preventing it from transactivating proinflammatory genes (Liberman *et al*., 2007). Inhibition of T-cell activation with Ca^{2+} channel blocking drugs also reduces T-bet expression, with attendant reduction in T-cell interferon-γ production (Di Sabatino *et al*., 2009).

Clearly, transcription factor programming of cellular behaviour is complex. A number of genes identified as T-bet binding targets by ChIP studies are at loci at which SNPs are significantly associated with IBD or coeliac disease in GWA studies. For instance, SNPs at loci encoding tumour necrosis factor superfamily 4, chemokine receptor 5 and *IL-18RAP* (Barrett *et al*., 2008; Dubois *et al*., 2010) are also genes specifically bound by T-bet in ChIP experiments (Gocke *et al*., 2007; Jenner *et al*., 2009).

1.4.1.6 T-bet deficiency in the innate immune system results in spontaneous IBD

Loss of T-bet confined to the innate immune compartment in mice lacking an adaptive immune system (*Rag2*$^{-/-}$ mice), results in spontaneous colitis resembling some aspects of human UC (Garrett *et al*., 2007). Like the human disease, TRUC (*Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ Ulcerative Colitis) mice develop a predominantly distal colitis. Histological appearances resemble human UC with predominantly superficial colonic inflammation, crypt abscess formation and infiltration of the colonic lamina propria with neutrophils and mononuclear cells (Garrett *et al*., 2007).

The inflammatory process is characterised by overproduction of TNFα in the lamina propria, which predominantly maps to CD11c^{+} class II^{+} myeloid cell populations, including dendritic cells. Selective depletion of CD11c^{+} cells (Garrett *et al*., 2009), or TNFα blockade prevents disease (Garrett *et al*., 2007), and TRUC mice lacking the p55 component of the TNFα receptor (triple knockout *Tbx21*$^{-/-}$, *Rag2*$^{-/-}$, *Tnf1/p55*$^{-/-}$ mice) (Garrett *et al*., 2007) are resistant to disease.

In TRUC mice deregulated TNFα was shown to cause increased epithelial permeability and was shown to be directly toxic to colonic epithelial cells, in which it triggered apoptosis (Garrett *et al*., 2007). Importantly, early treatment with TNFα neutralizing antibodies was capable of reversing early disease in TRUC mice, however, if anti-TNFα mAbs were administered beyond 12 weeks of age they failed to control disease (Garrett *et al*., 2007; Garrett *et al*., 2009). As well as showing the importance of TNFα in TRUC disease, these data also indicate that alternative innate immune mechanisms are responsible for driving chronic disease.

Interestingly, Tregs were shown to suppress colitis in TRUC mice (Garrett *et al*., 2007). Adoptively transferred CD4^{+} CD62L^{high} CD25^{+} Tregs migrated to the colon of TRUC mice and fully inhibited colitis development. Tregs were observed in close proximity to DCs in the colon of TRUC consistent with the possibility that Tregs suppress colitis by controlling the inflammatory potential of DCs.

1.4.1.7 The TRUC model of IBD is dependent on the intestinal microbiota

Several lines of evidence indicate that the TRUC model of IBD is dependent on the colonic microflora. Colitis could be communicated to WT mice co-housed or cross-fostered with TRUC mice, leading to the hypothesis that host deficiency of *Tbx21* somehow encouraged the establishment and expansion of a colitigenic community of intestinal microbes that could subsequently be transmitted to other mice (Garrett *et al*., 2007). Treatment of TRUC mice with a combination of broad-spectrum antibiotics (vancomycin, metronidazole, neomycin and ampicillin), a mixture that has been shown to substantially deplete intestinal bacteria (Fagarasan *et al*., 2002), resulted in 100,000 fold depletion of culturable anaerobes and induced remission of disease (Garrett *et al*., 2007). Monotherapy with metronidazole or gentamicin also cured disease (Garrett *et al*., 2010). Probiotic supplementation in TRUC mice with a *Bifidobacterium lactis* containing bacterial mixture also reduced colitis (Veiga *et al*., 2010), further demonstrating how manipulation of the intestinal microbiota can alter the severity of colitis in genetically susceptible animals. Probiotic bacteria reduced the pH of the caecum of TRUC mice, prompted expansion of short-chain fatty acid producing bacteria and increased levels of butyric acid, acetic acid and proprionic acid in the caecum.

In 2010 Garrett *et al*. reported that *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ double knockout mice re-derived under germ free conditions failed to develop colitis, which demonstrated conclusively that environmental bacteria are absolutely required for disease penetrance in this model (Garrett *et al*., 2010). The authors reported the first comprehensive comparison of the intestinal microbiota between TRUC and *Rag2*$^{-/-}$ mice using culture independent sequencing 16S rRNA genes. In TRUC mice there was a relative expansion in the proportion of *Bacteroidetes* and a reduction in the proportion of *Firmicutes* (Garrett *et al*., 2010). The authors also performed extensive cultures of the faeces of TRUC and *Rag2*$^{-/-}$ mice and noted that the bacteria *Klebsiella pneumoniae* and *Proteus mirablis* were present in all TRUC mice, and that these bacteria were additionally observed to be sensitive to both metronidazole and gentamicin. However, inoculation of GF mice with these bacteria by themselves, or in combination, was unable to recapitulate disease, demonstrating that exposure to additional as yet unrecognised environmental stimulus was necessary to trigger colitis in T-bet deficient hosts.

1.4.1.8 TRUC mice develop inflammation associated colorectal cancer

In addition to sharing a number of important clinical features with UC, TRUC mice developed colonic epithelial dysplasia and adenocarcinoma due to long-standing uncontrolled colonic inflammation (Garrett *et al*., 2009). Carcinogenesis in the TRUC model recapitulates many phenotypic and molecular features of carcinogenesis in UC. By 3 months of age, half the colony has dysplasia, rising to 96% by six months of age. By that time, 42% have frank adenocarcinoma. Similar to carcinogenesis in UC, carcinoma in the TRUC mouse arises from mucosa with the longest-standing inflammation (the rectum), is typically flat and arises from areas of dysplasia. The TRUC epithelium is rich in reactive oxygen species and DNA adducts, the levels of which correlate with duration of colonic inflammation. The molecular pathogenesis of CRC in TRUC mice more closely resembles UC-associated CRC than sporadic CRC, with early loss of function of *p53*, early increased epithelial COX-2 expression and later *APC* mutations, leading to abnormal β-catenin localisation. Furthermore, unlike the cytokine signature observed in early TRUC colitis, that is dominated by TNFα expression, the inflammatory microenvironment associated with dysplasia and adenocarcinoma is also characterized by increased expression of other proinflammatory cytokines, such as IL-6, similar to the situation observed in UC associated CRC (Danese and Mantovani, 2010; Garrett *et al*., 2009).

Hypothesis and Aims

The key aims of this thesis are:

1 To identify the unknown innate immune mechanisms responsible for driving chronic intestinal inflammation in TRUC mice

2 To identify the microbial drivers of intestinal inflammation in TRUC mice

3 To define how T-bet might impact on relevant host-microbe interactions in gut

The hypotheses tested in this thesis are:

1 Innate lymphoid cells are emerging as key mediators of mucosal inflammation. This thesis will test the hypothesis that ILCs are responsible for mediating chronic IBD in TRUC mice.

2 Cytokines such as the IL-23/IL-17 axis, IL-6 and TL1A are strongly suspected to play a key role in IBD pathogenesis. Although these molecules are conventionally thought to drive adaptive mucosal responses, this thesis will test the hypothesis that these molecules are involved in driving innate immune mediated colitis.

3 Intestinal inflammation in TRUC mice is dependent on the intestinal microbiota. This thesis will test the hypothesis that a particular intestinal bacterial species will be responsible for driving the innate immune mechanisms responsible for causing IBD in TRUC mice.

CHAPTER 2

Material and Methods

2.1 Animal husbandry

BALB/c *Tbx21*$^{-/-}$ (Jackson labs), *Rag2*$^{-/-}$ (Jackson labs) and *Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ (Taconic) mice were sourced commercially. A novel colony of colitis free *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ (TRnUC) mice was generated and remotely maintained at geographically distinct isolators from the TRUC colony that was descendant from the original described TRUC colony (Garrett *et al*., 2007). *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ *x Il2rg*$^{-/-}$ mice were generated internally and housed remotely. Mice were housed in Specified Pathogen-Free (SPF) conditions. All mice were handled according to both local (KCL) and national standards, and all experimental protocols were reviewed and approved by our local ethics review committee. All animal experiments were conducted in accredited facilities in accordance with the UK Animals (Scientific Procedures) Act 1986 (Home Office license number PPL 70/6792).

2.2 Genotyping

Genomic DNA was isolated by overnight digestion at 56°C of ear or tail samples in 200µl of lysis buffer (5 mM EDTA, 100 mM Tris-HCl pH 8.5, 0.2% SDS, 200 mM NaCl, 1mg/ml Proteinase K). Digested samples were diluted 1:4 in nuclease-free water. PCR reaction comprises 12.5µl 2x Mango Mix (Bioline Ltd., London, UK), 10µl nuclease-free water, 1 µl each of PCR primers (diluted in nuclease-free water to 25µM), 0.5µl genomic DNA. PCR products were mixed with Gel loading dye (Biolabs) and 10 µl loaded on a 1 % agarose gel premixed with GelRed Nucleic Acid Gel Stain (Biotium) to visualise DNA bands following UV illumination (Unipro Gold).

2.3 Tissue harvest and preparation

Mice were euthanized by inhalation of a rising concentration of carbon dioxide gas or by cervical dislocation, and then dissected in a laminar flow cabinet using aseptic technique. Spleen and mLN were excised and placed in ice-cold complete cell culture medium. Colon was excised and prepared by gentle expulsion of colonic contents, followed by flushing with ice-cold sterile PBS. Mechanical disruption of spleen and mLN through nylon mesh was performed to prepare a single cell suspension suitable for further manipulation. Cell suspensions isolated from spleen (but not mLN) were subjected to red blood cell lysis by resuspension for 2 minutes in ACK lysis buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4). All cell washing and resuspension was performed with complete culture medium (see below) unless otherwise stated and trypan-blue staining was used to assess cell viability and count live cells.

2.4 Isolation of colonic lamina propria leukocytes

Colons were cut longitudinally, faecal material removed and washed with PBS. Colons were then cut in to 5 mm segments and the epithelium removed by incubating with HBSS without Mg^{2+}/Ca^{2+} (Invitrogen) supplemented with 10% FCS foetal calf serum gold (FCS) (PAA Laboratories), 10mM HEPES (Fisher Scientific), 100IU/mL penicillin and 100μg/mL, streptomycin (Invitrogen) and EDTA (5mM). Epithelial removal step was performed for 20 minutes at 37^{o}C with constant agitation in a shaking water bath. Cells from the epithelial layer (present in the supernatant) were discarded and the remaining epithelia-free colon fragments were captured using nylon mesh. Tissue was mechanically disrupted using GentleMACs (Miltenyi) using program C (45 second disruption) in 10mL of digestion buffer which comprised complete medium (see below) supplemented with 25μg/mL collagenase D (Roche), 10μg/mL DNAse I (Roche) and 1.5mg/mL dispase (Roche). Following mechanical disruption in the GentleMACs the crude tissue/cell mixture was incubated in digestion buffer for 45 minutes in a shaking water bath (37^{o}C). 40mL of complete medium was added to the unfractionated crude cell mixture which was pelleted by centrifugation for 5 minutes at 652g. This process of resuspending cells in medium or PBS followed by 5 minutes centrifugation at 652g is subsequently referred to as

cell "washing". Cells were resuspended in 40% Percol (GE Healthcare) and layered on 80% Percol. Centrifugation was performed for 20 minutes at 805g without brakes at 20^{o}C. The cLPL enriched population was harvested from the interface between the 40% and 80% Percol using a Pasteur pipette. Cells were washed in complete medium and stored on ice pending downstream analysis.

2.5 Cell culture

Unfractionated splenocytes ($2x10^6$/mL), mLN ($1x10^5$/ml) and cLP cells ($1x10^5$/mL) were cultured in complete medium: RPMI-1640 medium (PAA Laboratories), supplemented with 10% FCS (PAA Laboratories), 50μM 2-mercaptoethanol (Gibco), 2μM L-glutamine (Sigma), 1mM sodium pyruvate (Sigma), 10mM HEPES (Fisher Scientific), non-essential amino acids (Sigma), 50IU/mL penicillin and 50μg/mL streptomycin (Sigma), (subsequently referred to as complete medium). Cells were cultured in medium alone or in the presence of recombinant murine cytokines, including IL-23 (20ng/mL), TNFα (20ng/mL), IL-6 (50ng/mL) and TL1A (50ng/mL or 100ng/mL) (R&D systems). Supernatants were harvested and cytokines concentrations measured by ELISA.

2.6 Immunomagnetic cell isolation

Enrichment of $CD11c^+$ cells from the cLP was achieved by positive selection using immunomagnetic cell purification. Unfractionated cLPLs were washed in PBS and resuspended in MACs buffer (PBS, 5mM EDTA, 1% FCS). Cells were incubated with anti-CD11c microbeads for 20 minutes at 4^{o}C. Excess beads were removed by centrifugation according to the manufacturer's instructions and microbead-labelled cells were resuspended in MACS buffer before being and passed through MACS LS columns mounted on a quadroMACS magnet. Positively selected cells were eluted and washed in complete medium and stored at 4^{o}C pending downstream analysis/use. All reagents and equipment supplied by Miltenyi Biotec Gmbh, Bergisch Gladbach, Germany. In some instances anti-PE beads were used following cell labelling with PE-conjugated mAbs (e.g. anti-F4/80 and CD11b).

2.7 *Ex vivo* organ culture

3mm punch biopsies (Miltex) were used to acquire full thickness colonic biopsies. 3 biopsies were cultured in 300-500μL of complete medium for 48 hours. Cytokine concentrations in culture supernatants were measured by ELISA (R&D systems or eBiosceince).

2.8 Inhibition of STAT3 phosphorylation

STAT3 phosphorylation was inhibited *in vitro* using the soluble small molecule S3I-201(Merck, UK) at doses of 20μg/ml and 100μg/ml in explant culture and at 100μg/ml in splenocyte and mLN culture. S3I-201 was dissolved in 0.1% DMSO. Control wells were incubated in the presence of 0.1% DMSO alone. Changes in cytokine production in culture supernatants were measured by ELISA.

2.9 ELISA

Cytokine concentrations were measured in culture supernatants by ELISA. Samples were measured in duplicate and standard curves created with serial dilutions of standards provided according to the manufacturer's instructions. Cytokine concentrations in samples were calculated by interpolation from the standard curve in the linear phase of the curve using standards (recombinant cytokine) of known concentration provided by the supplier. ELISA kits were purchased from R&D systems unless otherwise stated for the following cytokines; IL-17A, interferon-γ, IL-22, TNFα and IL-6 (eBioscience).

2.10 Flow cytometry

Flow cytometry was performed on unfractionated splenocytes, mLN or cLPL cells. In experiments where cells were stimulated prior to performing flow cytometry, $1x10^6$ cells were plated in 48 well tissue culture plates in 1ml of complete medium. Cells were stimulated with 50ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 1μg/ml ionomycin (Sigma), or IL-23 (10-20ng/ml), IL-6 (50ng/mL), TL1A (20-100ng/ml) or TNFα (20ng/mL) for 4 hours, adding 2μM monensin (Sigma) for the final 2 hours to inhibit intracellular protein exportation. To analyse intracellular

TNFα production by cLP DCs, unfractionated cLPLs were cultured in the presence of monensin for 4 hours at 37^0C. A control population of cLPLs was incubated on ice without monensin to serve as negative controls and to facilitate the definition of flow cytometry gates showing TNFα producing cells. After stimulation (and control incubation) cells were washed once in PBS and resuspended in 200µL of PBS containing ant-CD16/CD32 to block non-specific binding via fc receptors. Cells were washed in PBS and then resuspended in 200µL of PBS containing appropriate surface staining monoclonal antibodies together with cell viability stain (LIVE/DEAD® fixable Aqua dead cell stain kit, Life Technologies) for 20 minutes at room temperature covered in foil. This viability stain is an exclusion dye and is compatible with subsequent cell fixation. Antibody clones and fluorochromes used are listed in Table 4. After surface/viability staining cells were fixed by resuspending in 400 µL of 2% paraformaldehyde for 20 minutes at room temperature. After fixation surface cells were washed in permeabilisation buffer (eBioscience) by adding 1mL of permeabilisation buffer directly to the paraformaldehyde suspended cells. After washing cells were resuspended in 200 µL of appropriate intracellular staining monoclonal antibodies in 1x permeabilisation buffer for 30 minutes at 4^0C. Cell were washed in PBS and resuspended in 400 µL of PBS and stored at 4^oC in the dark pending acquisition. Cells were acquired within 24 hours of staining.

Samples were acquired using identically configured BD LSR II and BD LSR Fortessa (BD Biosciences) flow cytometers (20 parameter SORP instrument, 355 nm (nUV), 405 nm (violet), 488nm (blue), 561nm (Yellow-green), 633 nm (red)), allowing simultaneous use of all lasers. Sample data were recorded in FCS 3.0 data format using BD FACSDiva 6.0 software (BD Biosciences). Sample analysis was performed using FlowJo software (Treestar Inc., Ashland, OR, USA).

2.11 Flow cytometry compensation calculation

Calculating fluorescence compensation was first achieved using the automated compensation calculator programme in BD FACSDiva. Compensation set up was achieved using anti-mouse or anti-rat compensation beads (BD^{TM} CompBeads, BD Biosciences) labelled with a single fluorochrome conjugated monoclonal antibody. Compensation beads were coupled to the same fluorochrome conjugated antibodies

as those used for the specific stains used to label cells in the same experiment. Compensation beads were labelled with the appropriate fluorochrome conjugated monoclonal antibody for 15 minutes at room temperature. In early experiments unstained cells were used in the compensation set up to define the absence of staining. In later experiments negatively stained compensation beads were used. Automatically calculated spectral overlap values were manually inspected and the emission spectra of each single stained control analysed for spill over in to other fluorochrome channels. Where necessary compensation was manually corrected/fine tuned by adjusting automated compensation values.

2.12 Flow cytometry gating strategies

Positively stained cell populations were defined by comparing cell populations stained with an appropriate specific antibody with analogous cell populations stained with species/isotype matched fluorochrome conjugated control antibodies. For intracellular cytokine staining in experiments employing stimulation (e.g. PMA and ionomycin), the main control used to define positive staining was an analogous population of unstimulated cells that were treated with monensin alone. In these experiments cell populations were stimulated with PMA and ionomycin for 4 hours and monensin (2μM, Sigma) added for the final 2 hours of stimulation. In the unstimulated cells monensin alone was added for the final 2 hours of the incubation. In flow cytometry experiments the "monensin only" population of cells was used to identify the "negative" population of cells based on the assumption that without stimulation the cells would not produce any cytokine. In some experiments it was clear that a significant minority of cells stained positive for intracellular cytokine even in the absence of exogenous *in vitro* stimulation (e.g. Figure 6). In these cases an additional isotype control arm to the experiment was used. In isotype control experiments cell surface labelling antibodies were used in the same manner as in the stimulated cells stained with specific anti-cytokine monoclonal antibody, however, an isotype matched control antibody conjugated with a matched fluorochrome was used instead. Using this strategy cytokine negative populations were defined.

2.13 Flow cytometry cell sorting of ILCs

In some experiments ILCs from TRUC and *Rag2*$^{-/-}$ mice were purified from mLN by FACs. mLNs were used as a rich source of ILCs since target cells can be rapidly and easily isolated. Unfractionated mLN cells were harvested and a single cell suspension generated by mechanically disrupting cells through nylon mesh in complete medium. Unfractionated mLN cells were then labelled with 10µL of anti-CD90 (PE) and 10µL of anti-NKp46 (APC) for 15 minutes at room temperature together with the viability stain (AmCyan) in a volume of 300µL of PBS. Cells were washed in PBS and resuspended in 500µL of PBS. Cells were sorted on a FACSAria™ II Cell Sorter (BD Biosciences). Cells were initially gated on the lymphocyte gate identified by forward and side scatter. Live cells were identified by gating on the unstained population of cells using the viability stain. Subsequently, NKp46^{-} (to exclude NK cells), CD90^{+} cells (ILCs) were identified and collected into sterile medium pending analysis.

Table 4. Monoclonal antibodies and fluorochromes used in flow cytometry experiments

Antigen	Clone	Fluorochrome	Provider
CD45	30-F11	Pacific blue/AX700	eBioscience
CD4	RM4.5	PECy7	eBioscience
CD11b	M1/70	FITC/Pacific Blue	eBioscience
CD11c	N418	PE/APC Cy7	eBioscience
CD103	2E7	PE	eBioscience
F4/80	BMP	PE/PECy7	eBioscience
CD90.2	30H12	PE/FITC/Pacific Blue	eBioscience
NKp46	29A1.4	APC/FITC	eBioscience
CD127	A7R34	PE/Pacific Blue	eBioscience
RORγt	AFKJS-9	PE	eBioscience
CCR6	140706	PE	R&D systems
Sca-1	D7	AX700	Invitrogen
Gr-1	RB6-8C5	APC	eBioscience
IL-6R	D7715A7	PE/APC	BioLegend
DR3	Polyclonal	Biotin	R&D systems
T-bet	eBio4B10	PE/APC	eBioscience
α-IFNγ	XMG1.2	FITC/Pacific Blue/PE	eBioscience
IL-17A	eBio17B7	PE/FITC	eBioscience
TNFα	MP6-XT22	PE	eBioscience
IL-22	IH8PWSR	PE	eBioscience

2.14 RNA extraction

RNA extraction was performed using Trizol reagent (Invitrogen) according to the manufacturer's instructions, within a dedicated RNA work area. Equipment and surfaces were cleaned with RNaseZap solution (Ambion Inc., Austin, TX, USA) to reduce potential nuclease contamination. Cells were pelleted and resuspended in 1ml Trizol. Samples were either processed immediately or stored at -80°C prior to extraction. Whole colon segments (0.5-1cm) were harvested immediately after culling experimental animals and snap frozen in liquid nitrogen and then stored at -80^{o}C pending RNA extraction. Frozen colon segments were then homogenised using a Tissue lyzer II (Qiagen) with a Stainless Steel Beads (5 mm) (Qiagen) set to 25Hz/s for 5 minutes in 1mL Trizol reagent (Invitrogen). Cells or tissue homogenates were then immersed in 200µl chloroform (Sigma Aldrich), with the sample then mixed thoroughly and incubated at room temperature for 2 minutes. This was then centrifuged at 12,000g for 15 minutes at 4°C to allow phase separation. The upper colourless (aqueous) phase was carefully transferred to a fresh tube without disturbing the interphase, and 500µl of ice-cold isopropanol was then added. After vortex-mixing, the sample was centrifuged for 15 minutes at 4°C, at which point a pellet was observed. The supernatant was carefully removed; the pellet was then washed with 1ml 75% ethanol and centrifuged for a further 5 minutes at 7500g. The supernatant was then removed and the pellet allowed to air dry prior to resuspension in an appropriate volume of nuclease-free water. Samples were then assessed for quality, contamination and RNA concentration by analysis with a NanoDrop spectrophotometer. RNA was then stored at -80°C pending further analysis.

2.15 cDNA synthesis and quantitative PCR

cDNA was generated with the cDNA synthesis kit (Bioline) according to the manufacturer's instructions. Quantitative PCR was used to quantify mRNA transcripts using TaqMan gene expression assays (Applied Biosystems, Warrington, UK). Gene expression was normalized to the expression of β-actin to generate ΔCT values and relative abundance quantified using the $2^{-\Delta CT}$ method. The following Taqman qPCR primer sets were used according to the manufacturer's instructions; IL-17A (Mn00439619), IL-22 Mn00444241), IL-21 (Mn00517640), IFN-γ

(Mn01168134), TNF (Mn00443258), IL-4 (Mn99999154), TL1A (Mn00770031), IL23p19 (Mn00518984), IL12p40 (Mn00434165) and the housekeeping gene β-actin (4352341E).

2.16 Histology and colitis scores

Five to ten millimetre segments of distal (within 0.5cm of the anal verge) and in some experiments proximal colon were fixed in 10% paraformaldehyde, and embedded in paraffin blocks. 5μm sections were stained with haematoxylin and eosin. Colon histology preparation was performed by the Pathology Core at QMUL. Colitis scores comprising epithelial hyperplasia (0-3), epithelial injury (0-3), polymorphonuclear infiltrate (0-3) and mononuclear infiltrate (0-3) were reported in a blinded fashion (TTM) as described previously (Garrett *et al*., 2007). In most of the experiments reported in this thesis using TRUC mice the distal colitis score was used as one of the main experimental outcome readouts, since this is the area of the colon that appears to be preferentially affected from the macroscopic appearance of the colon (see Figure 4C). In the IL-6 blockade experiment the proximal colon was also scored histologically. This was the final *in vivo* blockade experiment performed during this thesis, hence proximal colon histology segments were not taken for analysis in the other *in vivo* experiments. The recognition that colitis is more severe in the distal colon in comparison with the proximal colon in this experiment did result in conducting an experiment to formally show the colitis scores in the distal and proximal colon of TRUC mice (Figure 5C).

2.17 Immunofluorescence

Fluorescent immunohistochemistry was performed in collaboration with Dr. Ellen Marks as follows. Tissue samples were snap frozen in Jung tissue freezing medium (Leica Microsystems). 7 μm cryostat sections were fixed in acetone and then blocked with 20% normal horse serum (PAA Laboratories). Sections were incubated with FITC-conjugated anti-CD90.2 (eBioscience). Nuclei were counterstained with 1μg/mL DAPI (Invitrogen). Images were acquired with an Olympus BX51 microscope using Micro-Manager software (Vale Laboratory, University of California San Francisco).

2.18 *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ Ulcerative Colitis (TRUC) model of colitis

The TRUC mice studied in this thesis were a kind gift from Professor Laurie Glimcher and were descendant from the originally described TRUC colony from Harvard University (Garrett *et al*., 2007). In view of the transmissibility of TRUC colitis, TRUC mice were housed in a separate isolator. TRUC mice develop spontaneous colitis and were vigilantly observed for adverse signs, including wasting, piloerection, intractable rectal prolapse or severe rectal bleeding. Animals developing these features or other adverse events were humanely culled on welfare grounds.

2.19 Anti-CD40 model of inflammatory bowel disease and systemic autoimmunity

One hundred and twenty-five µg of agonistic anti-CD40 mAb (clone FGK4.5) or rat IgG2a isotype control mAb (clone 2A3) (both from BioXCell, West Lebanon, US) were administered *i.p.* in a total maximum volume of 200µL in sterile PBS to *Rag2*$^{-/-}$, TRnUC or *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice. Mice were weighed at baseline and inspected on a daily basis for signs of disease including diarrhoea, rectal bleeding, clinical features of peritonism, hunched appearance and piloerection. Mice were sacrificed and examined between days 7-8 days post injection of anti-CD40. Following sacrifice organs were harvested and weighed, including colon, mLN, spleen and liver.

2.20 *In vivo* treatment of mice

Antibody treatments comprised: 500µg anti-TNFα (clone XT3.11, days 0, 3, 6, 9, 12, 15, 18, 21), 150µg anti-IL23p19 (G23-8, eBioscience, days 0, 4, 8, 13, 16), 450µg of anti-IL-17A (17F3, days 0, 4, 8, 11, 15, 18 and 21), 1mg anti-CD90.2 mAb (30H12, days 0, 7, 14, 21 and 28), 1mg anti-IL7R (A7R37, days 0, 3, 6, 9, 12, 15, 18, 21), 750µg anti-IL-6 (MP5-20F3, days 0, 4, 9, 14, 18, 23 and 27), 1mg anti-CD4 (clone GK1.5, days 0, 7, 14, 21 and 28) and 500µg anti-TL1A (a kind gift from Professor Aymen Al-Shamkhani, Southampton, days 0, 4, 8 and 14). Control isotype clones used were MOPC-21 (mouse IgG1), HRPN (rat IgG1), 2A3 (rat IgG2a), LTF-2 (rat IgG2b). Monoclonal antibodies were purchased from Bio X Cell (West Lebanon, US) unless otherwise stated and were administered *i.p* in sterile PBS. With regards to

the mAb doses and administration intervals used in the protocols for *in vivo* blockade/depletion, these were largely based on previously published protocols, such as CD90 depletion (Buonocore *et al*., 2010), CD4 depletion (Sonnenberg *et al*., 2011), IL-17A neutralization (Buonocore *et al*., 2010), IL-23p19 neutralization (Hagemann *et al*., 2008, Cornelissen *et al*., 2013), IL-6 neutralization (Starnes *et al*., 1990) and IL-7R blockade (Seki *et al*., 2007). TL1A neutralization was empirically based on advice from the supplier (Professor Al-Shamkani). Additional considerations included the amount of antibody purchased and the number of available age matched mice for treatment. In most cases experimental design permitted the use of antibody quantities at least equal to, or in most cases in excess of the amount used in previously published studies. The TL1A neutralizing antibody was potentially used in a suboptimal dosing regimen (for less time than other anti-cytokine treatments) in view of the limited supply of antibody. Accordingly, results from this experiment should be interpreted with this in mind.

2.21 454 pyrosequencing analysis of intestinal microbiota 16S rRNA genes

Bacterial 16S rRNA genes were kindly sequenced by Dr Alan Walker and Professor Julian Parkhill (Sanger Institute, Cambridge). DNA was extracted from frozen faecal pellets using the FastDNA® SPIN Kit. 16S rRNA gene PCR-amplicons were generated for Lib-L 454 Titanium sequencing using barcoded primers targeting the V3-V5 regions of the 16S rRNA gene. The primers and barcode sequences used are given in Table 4. PCR products were generated using AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen). PCR cycling conditions were as follows: 94ºC for 2mins followed by 20 cycles of 94ºC for 30secs, 53ºC for 30secs and 68ºC for 2mins. PCR products were then quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Raw sequences were passed through the PyroTagger pipeline (Kunin *et al*., 2010), which filters poor quality reads, clusters sequences into OTUs at 97% similarity and assigns taxonomic classifications to each OTU based on the GreenGenes (DeSantis *et al*., 2006) and SILVA (Pruesse *et al*., 2007) databases, and trimmed to a length of ~400 bases. Further taxonomic classifications for each OTU were obtained by using the mothur software package (Schloss *et al*., 2009) to classify

the sequences according to the RDP (Cole *et al*., 2009) and SILVA (Pruesse *et al*., 2007) databases.

2.22 Helicobacter PCR

Bacterial DNA was extracted from faeces using the QIAamp DNA stool minikit (Qiagen). *Helicobacter typhlonius* specific PCR was performed using primer pairs that have been shown to amplify a 122bp sequence of the 16S rRNA gene in *H. typhlonius*, but not other *Helicobacter* spp. (Feng *et al*., 2005). (5'-AGG GAC TCT TAA ATA TGC TCC TAG AGT-3', and 5' ATT CAT CGT GTT TGA ATG CGT CAA-3'). The PCR conditions used were as follows: 95^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30 seconds for 35 cycles. *Helicobacter* genus generic PCR was performed with the primer pair (5'-TAT GAC GGG TAT CCG GC-3 and 5'-ATT CCA CCT ACC TCT CCC A-3') (Beckwith *et al*., 1997). The 374bp PCR product generated by genus generic primers was recovered from acrylamide gel for subsequent DNA sequencing, which was performed by an outside vendor (Charles River Laboratories UK).

2.23 Bacterial growth conditions

Helicobacter typhlonius (CCUG 48335 T) was acquired from the culture collection of the University of Gothenburg. HT was grown and maintained either on blood agar plates (Oxoid, UK) containing 5% defibrinated horse blood (TCS Bioscience, Buckingham, UK) or in brain heart infusion broth supplemented with 1% yeast extract and 5% horse serum by Dr Ahmed Hashim at QMUL. Cultures were incubated in an anaerobic atmosphere consisting of 80% N_2, 10% H_2 and 10% CO_2 (Don Whitley Scientific, Shipley, UK). 1-5 x10^7 organisms were gavaged into selected mice as described in the text. Transfer of TRUC microbiota was achieved by homogenizing freshly harvested faecal pellets from adult TRUC mice in PBS. Solid matter was removed by low speed centrifugation and the resulting microbial suspension used for oral gavage. Each gavage recipients received PBS suspension corresponding to approximately 50% of an individual faecal pellet.

2.24 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). Non-parametric data were analysed using the Mann-Whitney U test and normally distributed data using the student T-test. Some samples were also analysed using paired T-tests where appropriate. Statistical significance was assumed if the p value was <0.05. Where appropriate non-parametric data was also expressed as medians and interquartile ranges (IQR).

Table 5. **Barcodes used for each 454-sequenced mouse faecal sample.** Faecal samples were acquired from 4x TRUC mice and 8x TRnUC mice. Bases in italics are the 454 Lib-L 'B' adaptor. Bases in bold are the 454 Lib-L 'A' adaptor.

Sample	Barcode and 16S rRNA gene primer
TRUC1	GACTGTGTCCGTCAATTCMTTTRAGT
TRUC2	GAGACTGACCGTCAATTCMTTTRAGT
TRUC3	GAGAGTGTCCGTCAATTCMTTTRAGT
TRUC4	GAGTAGTGCCGTCAATTCMTTTRAGT
TRnUC1	GAGTCTGTCCGTCAATTCMTTTRAGT
TRnUC2	GAGTGTGACCGTCAATTCMTTTRAGT
TRnUC3	GATCTGCACCGTCAATTCMTTTRAGT
TRnUC4	GATGTGCTCCGTCAATTCMTTTRAGT
TRnUC5	GACACTGTCCGTCAATTCMTTTRAGT
TRnUC6	GACAGTGACCGTCAATTCMTTTRAGT
TRnUC7	GACTAGAGCCGTCAATTCMTTTRAGT
TRnUC8	GACTCTGACCGTCAATTCMTTTRAGT

The primer sequences were as follows:

357F -*CTATCCCCTGTGTGCCTTGGCAGTCTCAG*ACTCCTACGGGAGGCAGCAG

926R–**CCATCTCATCCCTGCGTGTCTCCGACTCAG**-barcode_sequence-CCGTCAATTCMTTTRAGT

CHAPTER 3

Results: Defining the cellular and molecular determinants of chronic intestinal inflammation in TRUC mice

TRUC mice develop a spontaneous colitis that resembles aspects of human UC demonstrating that T-bet plays an important role in the maintenance of intestinal homeostasis, and that in the absence of T-bet innate immunity is sufficiently dysregulated such that spontaneous disease manifests. Despite the early work identifying an important role for TNFα in this model, the innate immune mechanisms responsible for maintaining chronic disease in TRUC mice are unknown. By investigating the innate immune mechanisms operating in chronic IBD in TRUC mice this thesis set out to provide new insights into how T-bet controls innate immunity in the gut.

One of the key immune mechanisms operating in early TRUC disease is deregulated colonic TNFα production by $CD11c^{+}$ cells and accordingly early stages of TRUC colitis can be reversed by blocking TNFα (Garrett *et al*., 2007). However, beyond 12 weeks of age the therapeutic benefit of anti-TNFα mAbs is lost (Garrett *et al*., 2009), indicating that alternative immune mechanisms are responsible for driving chronic disease. Therefore, to determine which other immune mechanisms were potentially involved in the evolution of chronic TRUC IBD, the intestinal and systemic immune response of TRUC mice aged >12 weeks old was investigated.

3.1 TRUC mice developed severe distal colitis resembling aspects of human UC

TRUC mice developed spontaneous colitis with rectal prolapse, distal colonic thickening and mesenteric lymphadenopathy (Figure 4A-C). In comparison with age matched *Rag2*$^{-/-}$ controls, they developed significantly increased colonic mass (Figure 4D) and splenomegaly (Figure 4E). Histologically, the colonic lesion was characterised by goblet cell depletion, crypt abscess formation and infiltration of the lamina propria with granulocytes and mononuclear cells (Figure 5A). Marked

epithelial hyperplasia was also a prominent feature of chronic TRUC IBD. Histological colitis scores, based on epithelial hyperplasia (0-3), mucosal injury (0-3), polymorphonuclear infiltration (0-3) and mononuclear cell infiltration (0-3) were significantly higher in TRUC mice in comparison with *Rag2*$^{-/-}$ controls (Figure 5B). Histological lesions were also noted to be more severe in the distal colon, with variable and less severe extension into the proximal colon (Figure 5C), as is the case in UC patients.

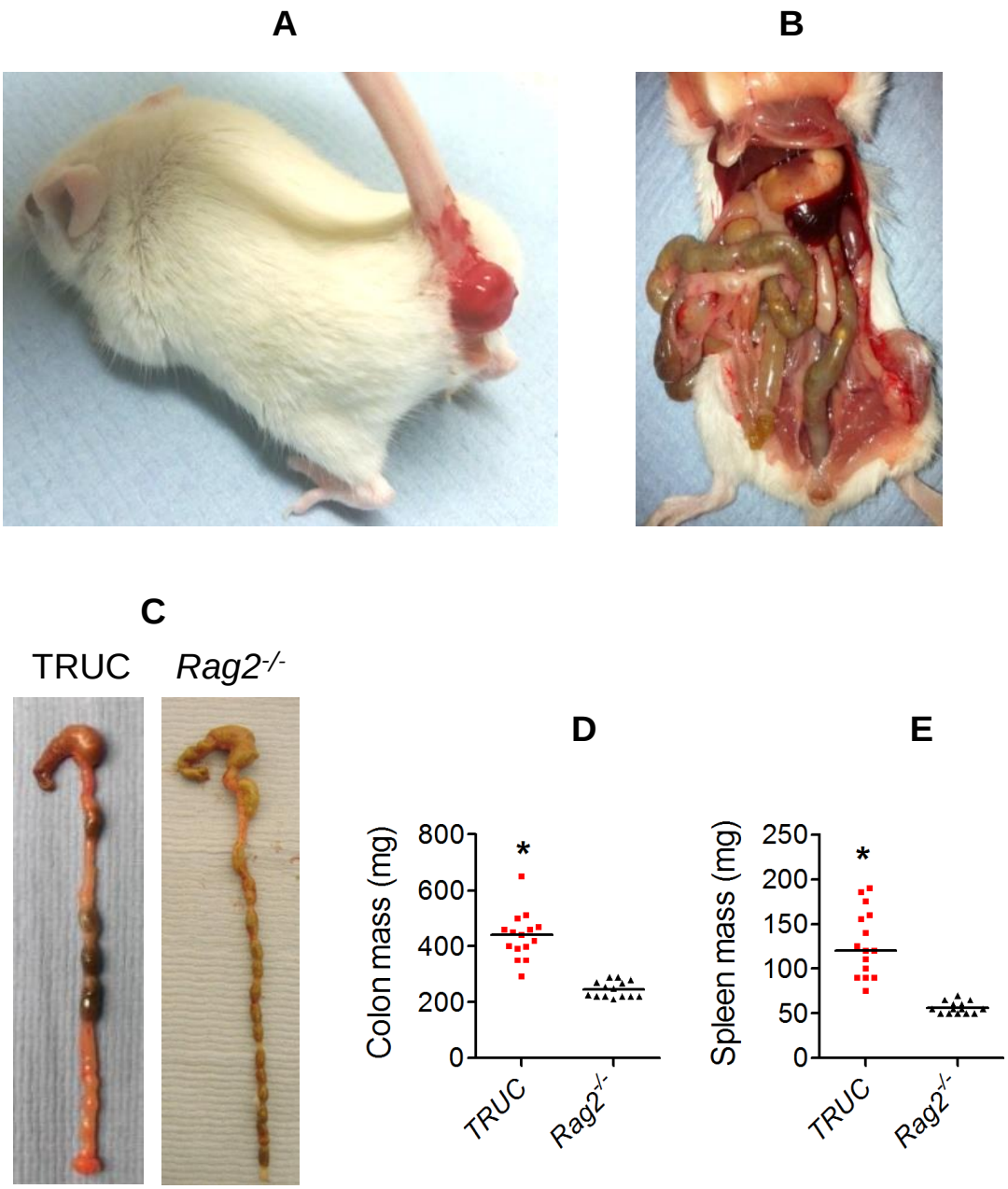

Figure 4. **The TRUC model of IBD.** A TRUC mice developed rectal prolapse. B Abdominal dissection demonstrated distal colonic thickening, splenomegaly and mesenteric lymphadenopathy. C The colon of TRUC mice was also markedly thickened, particularly distally, in comparison with *Rag2*$^{-/-}$ mice. D Colon and E spleen mass in 12 week old TRUC (n=15) and *Rag2*$^{-/-}$ (n=14) mice. *P<0.0001. Adapted from Powell *et al*., 2012.

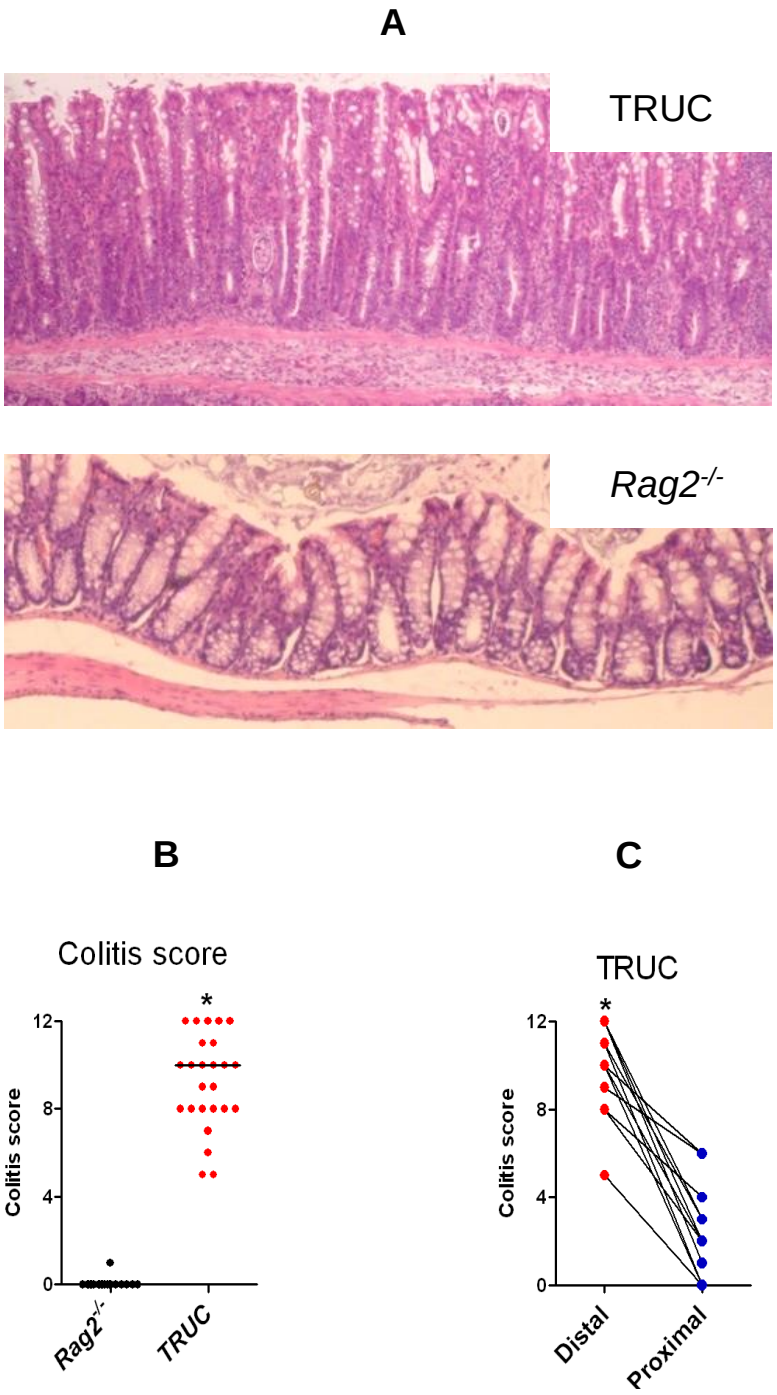

Figure 5. **Histological features of the colon in TRUC mice.** A Histological appearance of the distal colon (distal margin taken from within 0.5cm of the anal verge) of age matched (12 week old) TRUC and *Rag2*$^{-/-}$ mice (H&E stained micrograph). Magnification is the same for both panels (approximately x40). B Colitis histology scores from the distal colon in TRUC (n=25) and *Rag2*$^{-/-}$ mice (n=15) mice. *P<0.001. C Histology scores from the distal and proximal colon of 12-16 week old TRUC mice (n=15). *P<0.001. In Figure B each dot represents an individual mouse. In Figure C each pair of connected dots are from a single mouse.

3.2 Colonic CD45$^+$ immune cells from TRUC mice produced IL-17A and IL-22

To gain insights in to the potential immune mechanisms operating in chronic TRUC disease, cytokine production by colonic immune cells was investigated in mice >12 weeks old. Lamina propria leukocytes (LPLs) were isolated from the colon of TRUC mice by collagenase digestion and enrichment using density gradient centrifugation. Following purification, unfractionated cLPLs were stimulated with PMA and ionomycin to trigger cytokine production. In this experiment 2 additional controls were used. The first control (which was employed throughout the this thesis) used unstimulated cells treated with monensin alone. However, since there was sometimes a small amount of cytokine detectable in unstimulated cells treated with monensin alone it was also considered appropriate to use an additional control population of cells to appropriately define the negative and positive staining cell populations. This control was termed the isotype control. Cells were stimulated with PMA and ionomycin in the presence of monensin (added for the final 2 hours of the stimulation as in all other experiments). However, instead of adding the cytokine-specific intracellular mAb, an isotype-matched control mAb labelled with the same fluorochrome was added instead. In these experiments identical surface staining antibodies were used, thereby allowing isotype staining to be identified in specific cell populations.

Immune cell (CD45$^+$) expression of different cytokines potentially involved in mediating intestinal inflammation, including IFNγ, IL-4, IL-17A and IL-22 was then determined by intracellular flow cytometry. Following cell stimulation, cLPL were stained with a live/dead stain to identify viable cells and also with a surface antibody stain directed against CD45 to identify immune cells within the colon (Figure 6A). A control isotype matched antibody labelled with the same fluorochrome as the specific anti-CD45 mAb was used to define CD45$^+$ population within the colon. At the time of surface staining the unfractionated cells were also labelled with a viability exclusion dye that is compatible with cell fixation. Accordingly, unstained, live cells were defined and gated on for downstream analysis of intracellular cytokine. In comparison with unstimulated cells treated with monensin alone stimulation with PMA and ionomycin resulted in a marked increase in the number of cytokine

positive $CD45^+$ immune cells, particularly in the case of IL-17A and IL-22. Although few colonic $CD45^+$ cells expressed IFNγ or IL-4 even following activation with PMA and ionomycin, there were many cells expressing IL-17A and IL-22 (Figure 6C and 6D).

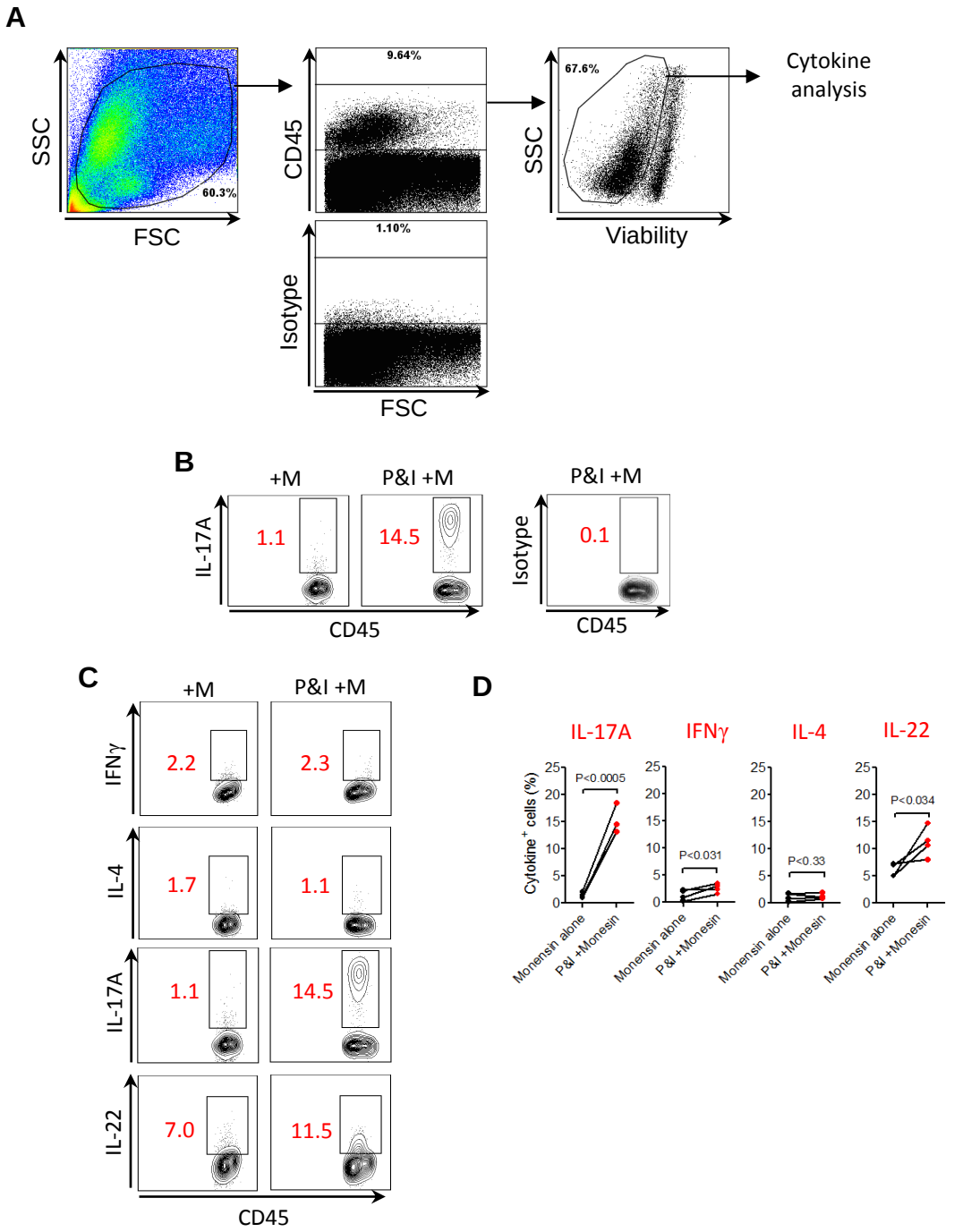

Figure 6. **Flow cytometric analysis of intracellular cytokine production by CD45$^+$ cLPLs from TRUC mice.** A Gating strategy employed to define CD45$^+$ cells by comparing specific anti-CD45 mAb staining with a matched fluorochrome conjugated isotype (bottom panel). Live cells were defined by the absence of LIVE/DEAD® fixable Aqua dead cell stain. B Gating strategy to define the proportion of cells (% of CD45$^+$ cells) positive for intracellular cytokine. Unstimulated cells treated with monensin alone (+M) were compared to cells stimulated with PMA and ionomycin with monensin (P&I +M). The positive cytokine gate was defined in stimulated cells stained with the same surface stain (CD45 and viability stain), but using an isotype control intracellular staining mAb. C Representative intracellular cytokine staining. D Statistical analysis (paired t-test) comparing the proportion of cytokine expressing cells in unstimulated /monensin alone cells compared to cells stimulated with P&I +M. Paired samples are from the same donor. Four biological replicates (individual mice) are shown. Adapted from Powell *et al.*, 2012.

Cytokine expression by $CD45^+$ immune cells was also measured in other tissues from TRUC mice, including the spleen and mesenteric lymph nodes (mLNs). Unfractionated splenocytes, mLN cells and cLPLs were stimulation with PMA and ionomycin and intracellular cytokine expression determined by flow cytometry. Although marked IL-17A expression was observed in both the cLP and mLN of TRUC mice, cytokine production by splenocytes was less pronounced (Figure 7), indicating that inflammatory cytokine production is enriched at the chief site of disease in TRUC mice, that is, in the colon and associated lymphoid tissue. Both colonic and mLN cells were a rich source of IL-17A.

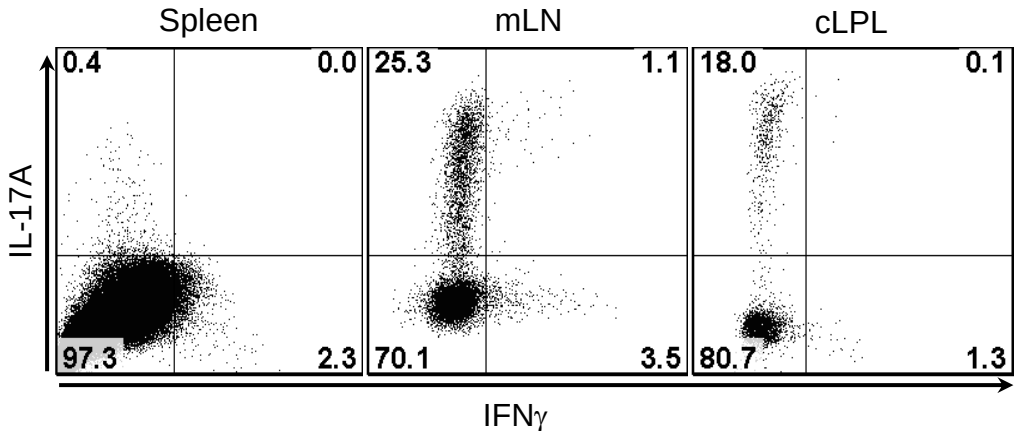

Figure 7. Cytokine expression by CD45$^+$ splenic, mLN and cLP immune cells in TRUC mice. Unfractionated splenocytes, mLN cells and cLPLs were stimulated with PMA and ionomycin. Intracellular expression of IFNγ and IL-17A was determined in live, CD45$^+$ cells in the tissues/organs specified (gating strategy as shown in Figure 6A). These flow cytometry plots are representative of >5 different experiments.

3.3 IL-17A production was increased in colon explant cultures from TRUC mice

Colon explant culture can be used to investigate cytokine production in disease relevant tissues. In these experiments full thickness colon was sampled from experimental animals. To standardise the size of colon assessed, 3mm wide biopsies were acquired from the distal colon of TRUC or *Rag2*$^{-/-}$ mice aged >12 weeks using a skin punch biopsy. Cytokines spontaneously secreted into culture supernatants were then measured following 48 hours of culture by ELISA. In comparison with *Rag2*$^{-/-}$ mice, explants from TRUC mice produced significantly more IL-17A and IL-22 (Figure 8). However, IFNγ and IL-4 production did not differ significantly between the genotypes.

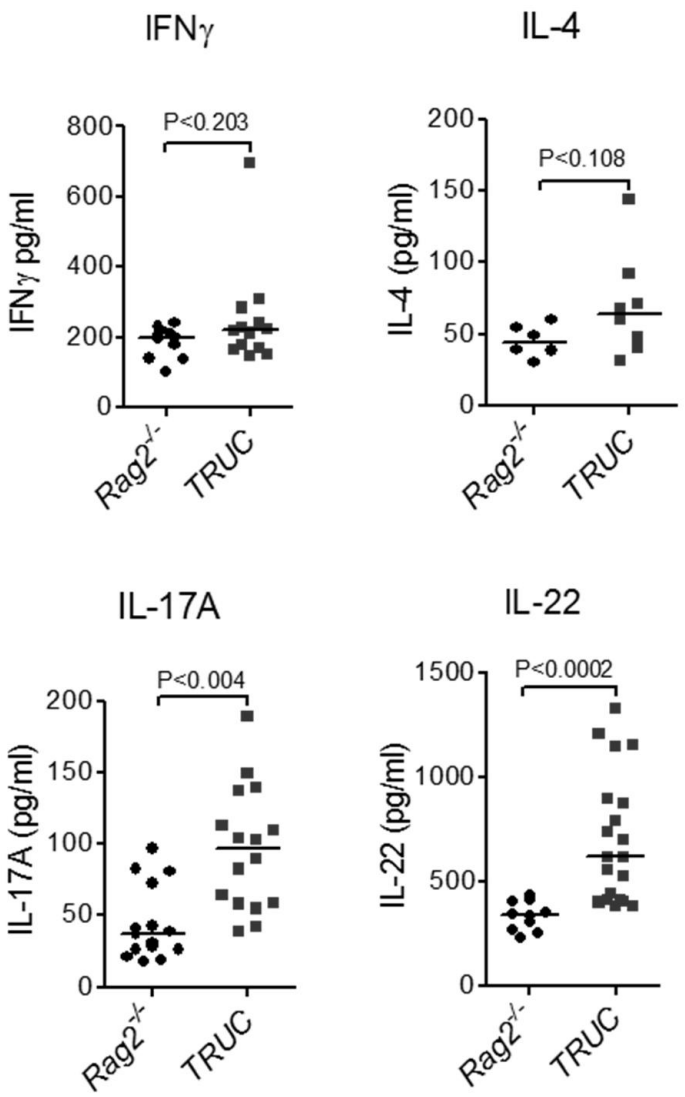

Figure 8. **Cytokine production in colon explant culture.** 3mm punch biopsies were taken from the distal colon of 12-16 week old *Rag2*$^{-/-}$ and TRUC mice. 3x biopsies were placed in 500µL of complete medium and incubated for 48 hours. Cytokine production was measured in supernatants by ELISA. Cytokine concentrations were interpolated from the linear part of the standard curve generated by measuring known standard concentrations of recombinant cytokines provided by the ELISA kit. Each data point represents an individual mouse. Lines depict median cytokine concentration.

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3.4 Transcripts encoding IL-17A were increased in the colon of TRUC mice

The expression of transcripts encoding inflammatory cytokines was also investigated in the colon of TRUC mice. 5mm segments of distal colon were harvested from TRUC and *Rag2*$^{-/-}$ mice into Trizol solution and stored at -80^{o}C. cDNA was then synthesized. Cytokine-specific primers and real time PCR were then used to quantify relative abundance of cytokine transcripts in colonic tissue. In agreement with flow cytometry and organ culture experiments, transcripts encoding *Il17a* and *Il22* were significantly increased in the colon of TRUC mice in comparison with *Rag2*$^{-/-}$ controls (Figure 9). However, there was no significant difference in the abundance of transcripts encoding *Ifng* or *Il4* in TRUC mice in comparison with *Rag2*$^{-/-}$ mice. Indeed, transcripts encoding *Ifng* were below the limit of detection in TRUC and *Rag2*$^{-/-}$ mice. In keeping with previous reports describing increased production of TNFα in TRUC disease (Garrett *et al*., 2007; Garrett *et al*., 2009), there was significantly increased abundance of transcripts encoding *Tnfa* in the distal colon of TRUC mice in comparison with *Rag2*$^{-/-}$ controls.

Since *Il17a* and *Il22* transcripts were significantly increased in TRUC mice, the possibility that other "Th17" like cytokines might also be increased in TRUC disease was considered. The cytokine IL-21 is also produced by Th17 cells and (Wei *et al*., 2007), and therefore the abundance of *Il21* transcripts in the colon of TRUC and *Rag2*$^{-/-}$ mice was also measured. Unlike transcripts encoding *Il17a* or *Il22*, which were elevated in TRUC mice, transcripts encoding *Il21* were not detected in the colon of most TRUC or *Rag2*$^{-/-}$ mice (Figure 9).

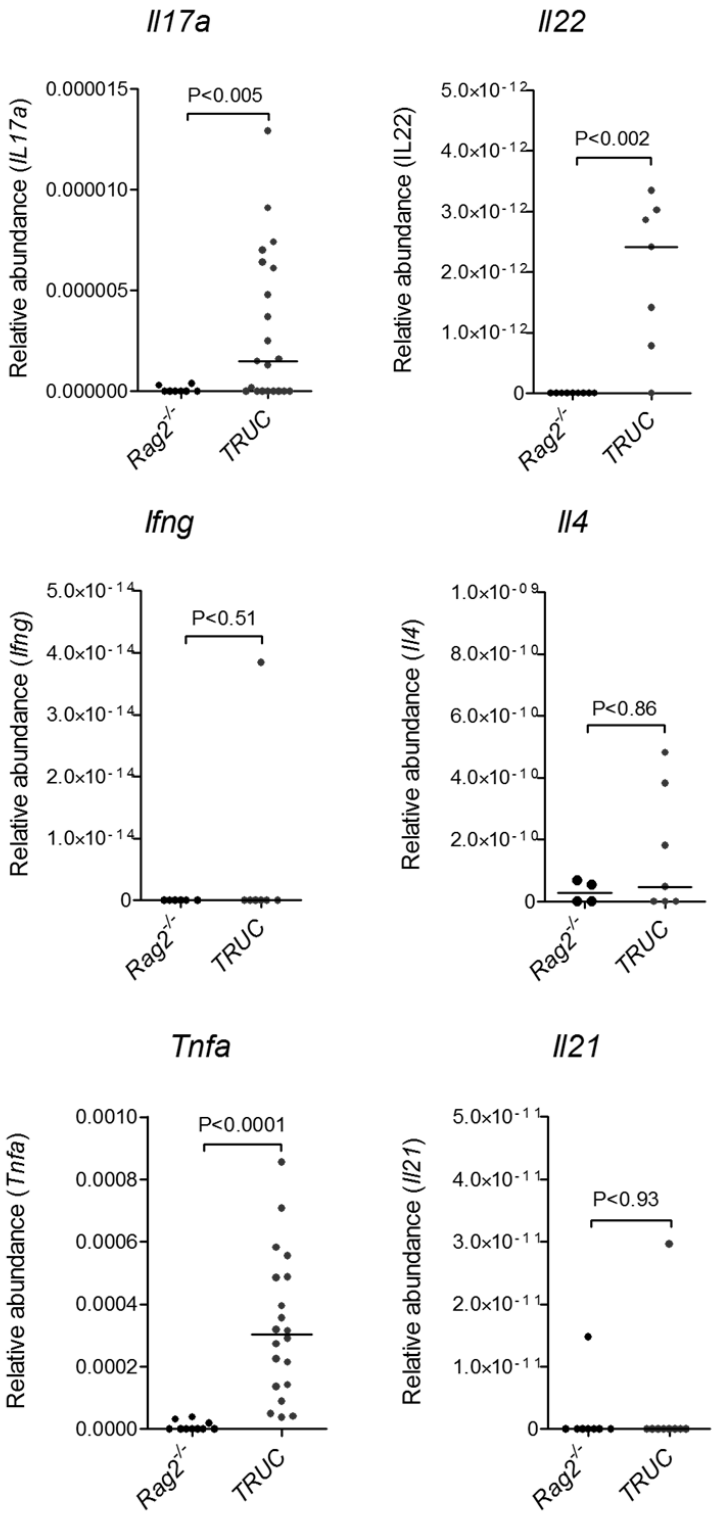

Figure 9. Quantification of cytokine transcripts in the colon of TRUC mice. RNA was extracted from 0.5cm segments of distal colon (within 0.5cm of anal verge) of *Rag2*$^{-/-}$ and TRUC mice. cDNA was synthesized and cytokine-specific PCR primers used to amplify corresponding transcripts. Line depicts median relative abundance of cytokine transcript. Each dot represents an individual mouse.

3.5 Innate lymphoid cells were the principle source of IL-17A in TRUC mice

Since excessive colonic production of IL-17A, a cytokine strongly implicated in IBD pathogenesis, was a prominent feature of chronic TRUC IBD, an attempt to define the cellular source of this cytokine was made. Innate sources of IL-17A have been reported to include ILCs (Buonocore *et al*., 2010; Takatori *et al*., 2009), NK cells (Hughes *et al*., 2009) and even neutrophils (Ferretti *et al*., 2003). To address this question, cells from TRUC mice were stimulated to produce IL-17A and then flow cytometry was employed, using antibodies directed against major lineage markers. Using flow cytometry it was observed that most of the IL-17A expressing cells present in the colon and mLN of TRUC mice were $CD90^{+}$ (Figure 10), a marker of ILCs (Buonocore *et al*., 2010). The expression of other cell surface innate immune cell lineage markers among the IL-$17A^{+}$ cell population was also investigated. There was no appreciable expression of NKp46 (NK cells), CD11c (myeloid cell lineages, including DCs), F4/80 (macrophages) and Gr-1 (granulocytes) among the IL-$17A^{+}$ cell population (Figure 11), indicating that other innate immune lineages were not responsible for significant IL-17A production in TRUC mice. Given that most of the IL-17A producing cells present in TRUC mice expressed CD90, the presence or absence of other ILC markers was determined in the IL-17A producing population of cells in TRUC disease. IL-$17A^{+}$ cells were observed to have increased expression of CCR6, RORγt, Sca-1 and IL-7Rα (Figure 11), consistent with the phenotype of ILCs (Buonocore *et al*., 2010; Sonnenberg *et al*., 2011). Taken together these data indicated that the excessive IL-17A production in TRUC IBD is derived from $CD90^{high}$ $CCR6^{+}$, RORγt^{+} IL-7Rα^{+} cells, consistent with the phenotype of ILCs, and that the other major innate immune lineages present in TRUC mice are not a significant source of IL-17A in this model.

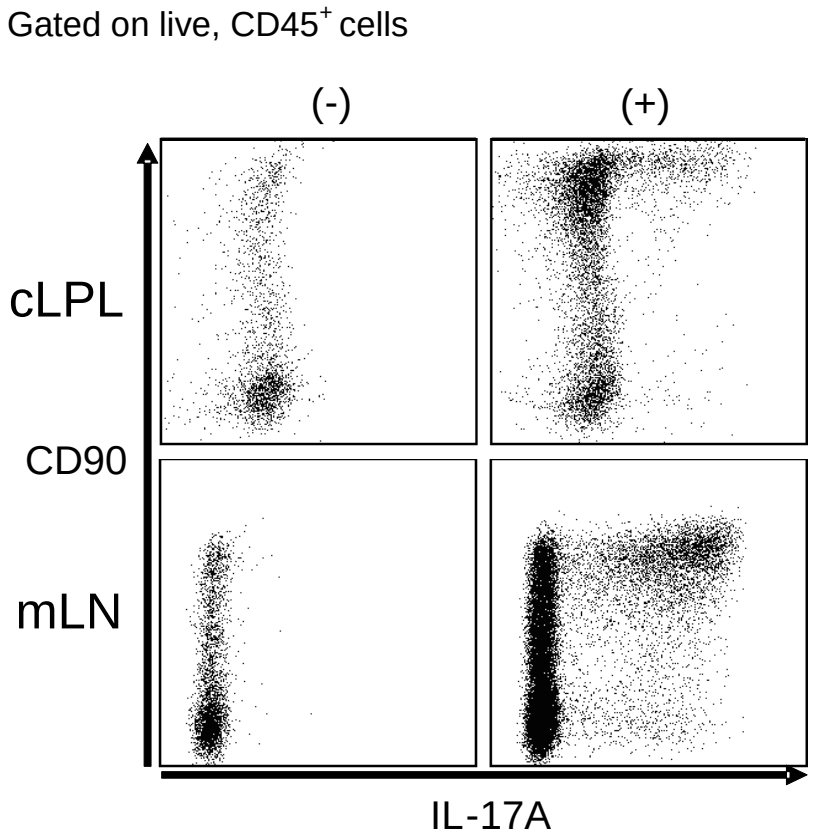

Figure 10. Most IL-17A producing cells in the colon and mLN of TRUC mice expressed the ILC marker CD90. Flow cytometry plot showing CD90 and IL-17A expression in unfractionated cLPLs and mLN cells following stimulation with PMA and ionomycin. These plots are gated on live, $CD45^+$ cells (as previously). These data are representative of many experiments (>10). Unstimulated cells cultured in the presence of monensin alone are denoted by (-) and cells stimulated with PMA and ionomycin in the presence of monensin are denoted by (+).

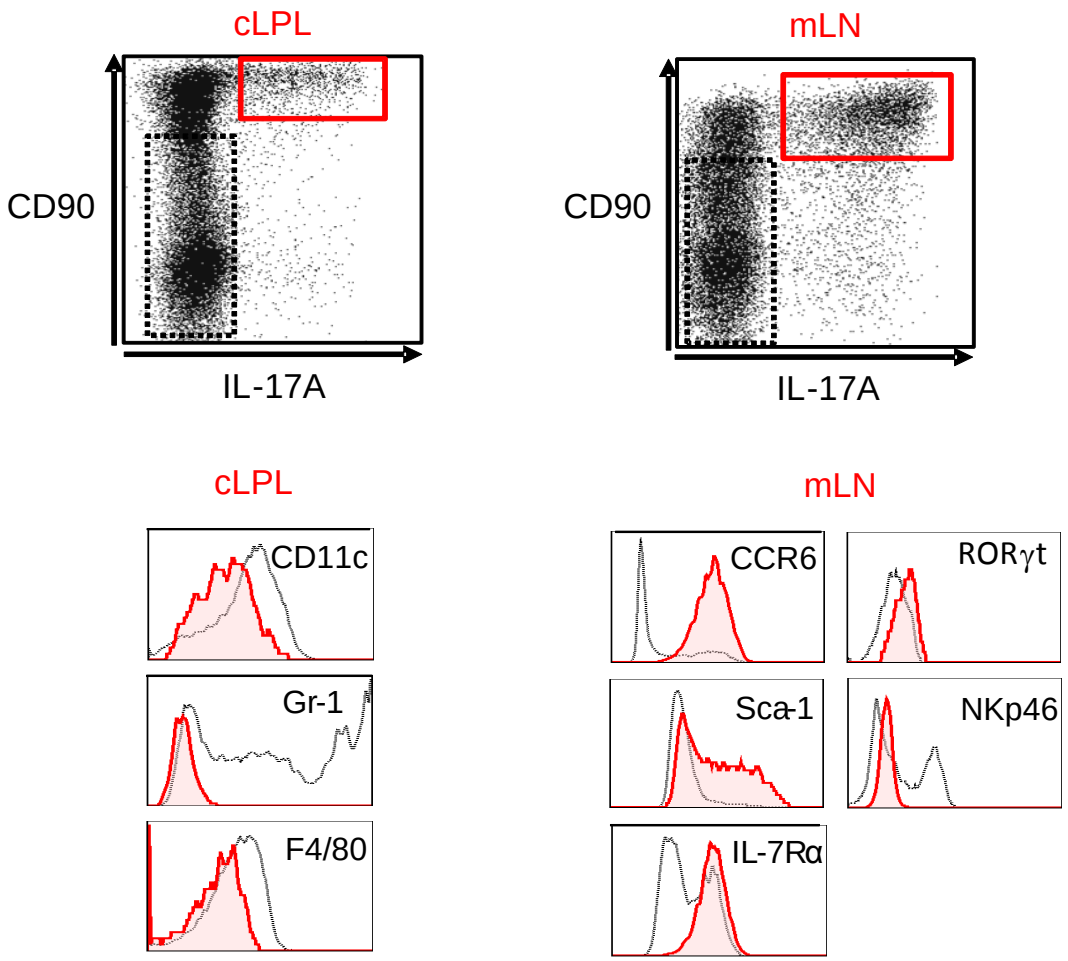

Figure 11. The phenotype of IL-17A expressing cells in TRUC colitis was consistent with the phenotype of ILCs. Unfractionated cLPLs (left panel) and mLN cells (right panel) were stimulated with PMA and ionomycin in the presence of monensin to define IL-17A producing cells. Positive staining was identified by comparing staining in unstimulated cells treated with monensin alone. Cells were stained for IL-17A and other cell surface markers, as shown. The expression of particular markers in the $CD90^{+}$ $IL\text{-}17A^{+}$ population (red histogram) and $CD90^{-}$ $IL\text{-}17A^{-}$ population (dotted line histogram) are shown. Data are representative of at least 2 individual experiments. Adapted from Powell *et al*., 2012.

3.6 Antibody mediated depletion of CD90 expressing cells reduced the number of IL-17A producing cells in TRUC mice

The chief cellular source of increased IL-17A production in TRUC colitis was CD90 ILCs. ILCs have been shown to play an important role in a number of mucosal disease settings (Buonocore *et al*., 2010; Sonnenberg *et al*., 2011) and therefore experiments were conducted to establish the functional significance of $CD90^+$ ILCs in TRUC IBD. The surface marker CD90 appears to be consistently expressed by all mucosal ILC populations, including the $CD4^+$ ILCs that contribute to host immunity to *Citrobacter rodentium* infection (Sonnenberg *et al*., 2011), $CD4^-$ ILCs that mediate inflammation in murine models of colitis (Buonocore *et al*., 2010), and also by nuocytes that express Th2 cytokines, such as IL-5 and IL-13 (Chang *et al*., 2011; Neill *et al*., 2010).

Therefore, to investigate the functional significance of $CD90^+$ ILCs in chronic TRUC colitis CD90 expressing cells were depleted *in vivo* in TRUC mice. In this experiment 1mg of depleting anti-CD90 (clone 30H12) or control antibody (rat IgG2b, clone LTF-2) was administered *i.p.* to 12-14 week old TRUC mice, once weekly for 4 weeks (Figure 12). This antibody clone has previously been to shown to successfully deplete colonic ILCs (Buonocore *et al*., 2010; Sonnenberg *et al*., 2011). Five mice received the control antibody and 4 mice received the depleting anti-CD90 antibody. At the end of the experiment (48 hours after the administration of final dose of depleting antibody) organs including the colon, spleen and mLN were harvested from study animals and analysed.

The total number of mononuclear cells present in different tissues of anti-CD90 and control mAb treated TRUC mice was calculated. Colons were harvested from study animals and cLPL isolated by collagenase digestion. Mononuclear cells were also harvested from the mLN and spleen following mechanical dissociation of the tissue. The total number of mononuclear cells present in each of the organs of study animals was then calculated. The number of splenocytes was significantly reduced in anti-CD90 treated mice (median = 4.7 x $x10^6$, inter-quartile range (IQR) 4.3-5.8) in comparison with control antibody treated mice (median = 10.1 $x10^6$, IQR 9.6-12.7, $P<0.016$). Similarly, the total number of mononuclear cells present in the mLN of anti-CD90 treated mice was also significantly reduced (median = 0.8 x 10^6, IQR 0.7-

0.8 x10^6) in comparison with control mice (median = 1.1 x 10^6, IQR 1.3-8.9 x10^6, P<0.02). There were also fewer cells present in the cLP of anti-CD90 treated TRUC mice (median = 1.05 x10^6, IQR 0.9-2.7 x10^6) in comparison with control mice (median = 2.2 x10^6, IQR 1.1-3.9 x10^6), however, this did not achieve statistical significance (P=0.323).

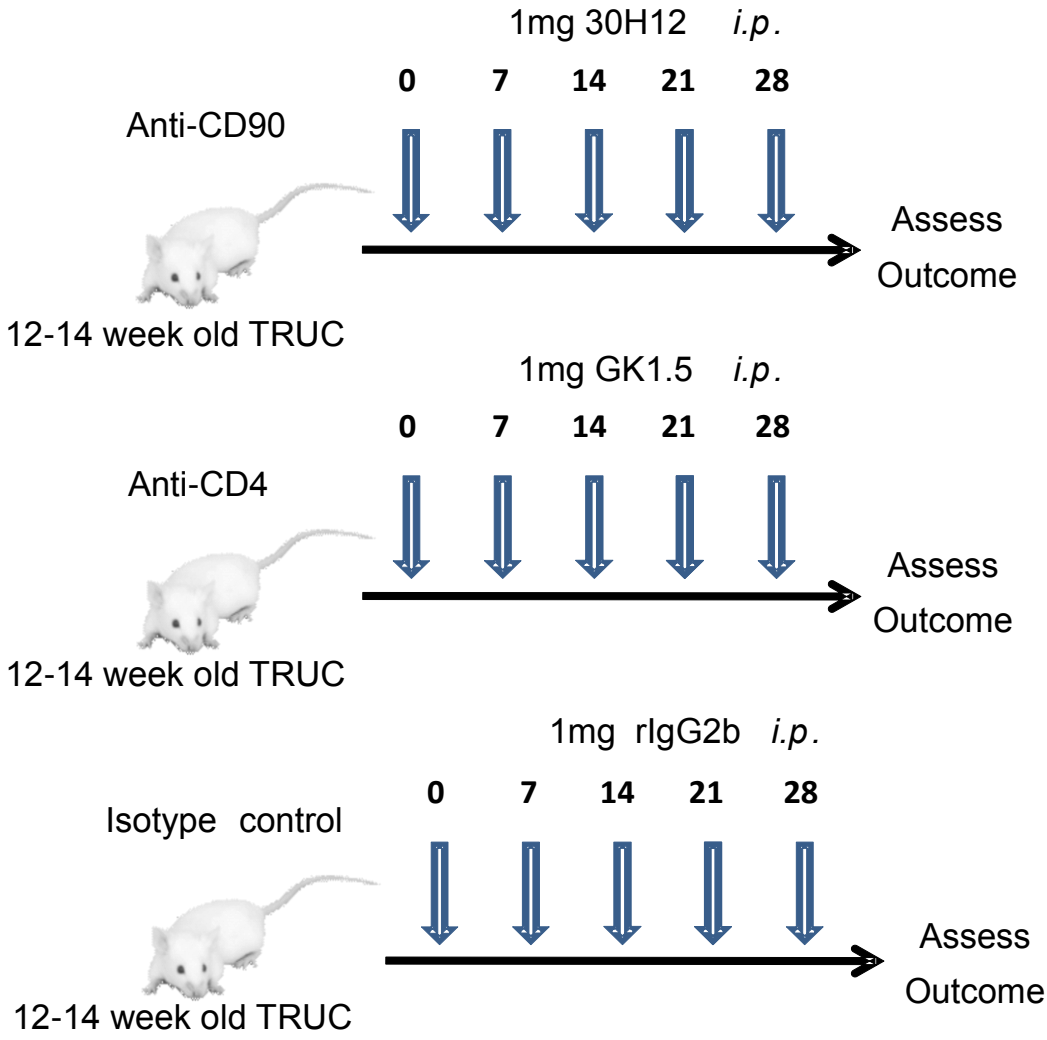

Figure 12. Protocol for *in vivo* depletion of CD90 or CD4 expressing cells in TRUC mice. 1mg of anti-CD90 (clone 30H12), 1mg anti-CD4 (clone GK1.5) or control antibody (clone LTF-2) was administered to 12-14 week old TRUC mice on days 0, 7, 14, 21 and 28. Mice were culled and organs harvested on day 30 (48 hours after the final dose of antibody). The CD90 depletion protocol was informed by previously published data regarding *in vivo* ILC depletion in the colon (Buonocore *et al*., 2010). In view of the paired anti-CD4 treatment, the GK1.5 was used at the same dose and timing. Previous studies have used comparable doses (e.g. 250µg every 3-4 days, Sonnenberg *et al*., 2011).

The number of $CD90^+$ $IL\text{-}17A^+$ expressing cells in the cLP and mLN of TRUC mice was also determined by flow cytometry. In this experiment, unfractionated cells were first stimulated with PMA and ionomycin to enable identification of IL-17A producing cells. Prior to intracellular staining (for IL-17A), cells were also stained with an anti-CD90 mAb. Crucially, administration of anti-CD90 resulted in marked depletion of $CD90^+$ cells in TRUC mice. A representative flow cytometry plot is shown in Figure 13A. As well as marked depletion of $CD90^{high}$ cells in both the cLP and mLNs of TRUC mice, the majority of IL-17A producing cells were also depleted following anti-CD90 administration, consistent with initial data showing that $CD90^+$ cells were the major source of IL-17A in TRUC mice. The absolute number of IL-17A producing cells in the cLP and mLN was also calculated by multiplying the proportion of $IL\text{-}17A^+$ cells in the cLP and mLN of the study TRUC mice by the total number of live mononuclear cells isolated from the tissues of each animal. In keeping with $CD90^+$ ILCs being the chief source of IL-17A in TRUC colitis, CD90 depletion resulted in a significant reduction in the absolute number of $IL\text{-}17A^+$ cells both the cLP ($P<0.02$) and mLN ($P<0.016$) of TRUC mice (Figure 13B).

The impact of CD90 cell depletion on colonic expression of transcripts encoding IL-17A in TRUC mice was also investigated. *Il17a* mRNA was quantified by qPCR following treatment with anti-CD90 or control antibody. Although there was a reduction in the median relative abundance of *Il17a* mRNA in the 5 anti-CD90 treated TRUC mice in comparison with the 4 control antibody treated animals, this did not achieve statistical significance (Figure 13C).

In addition to using flow cytometry, CD90 expressing cells were also visualised in the colon of TRUC mice by immunofluorescence (Figure 14). In anti-CD90 treated mice there were very few $CD90^+$ cells seen in the colon. In contrast, $CD90^+$ cells were frequently observed in the colonic lamina propria of TRUC mice given the control antibody.

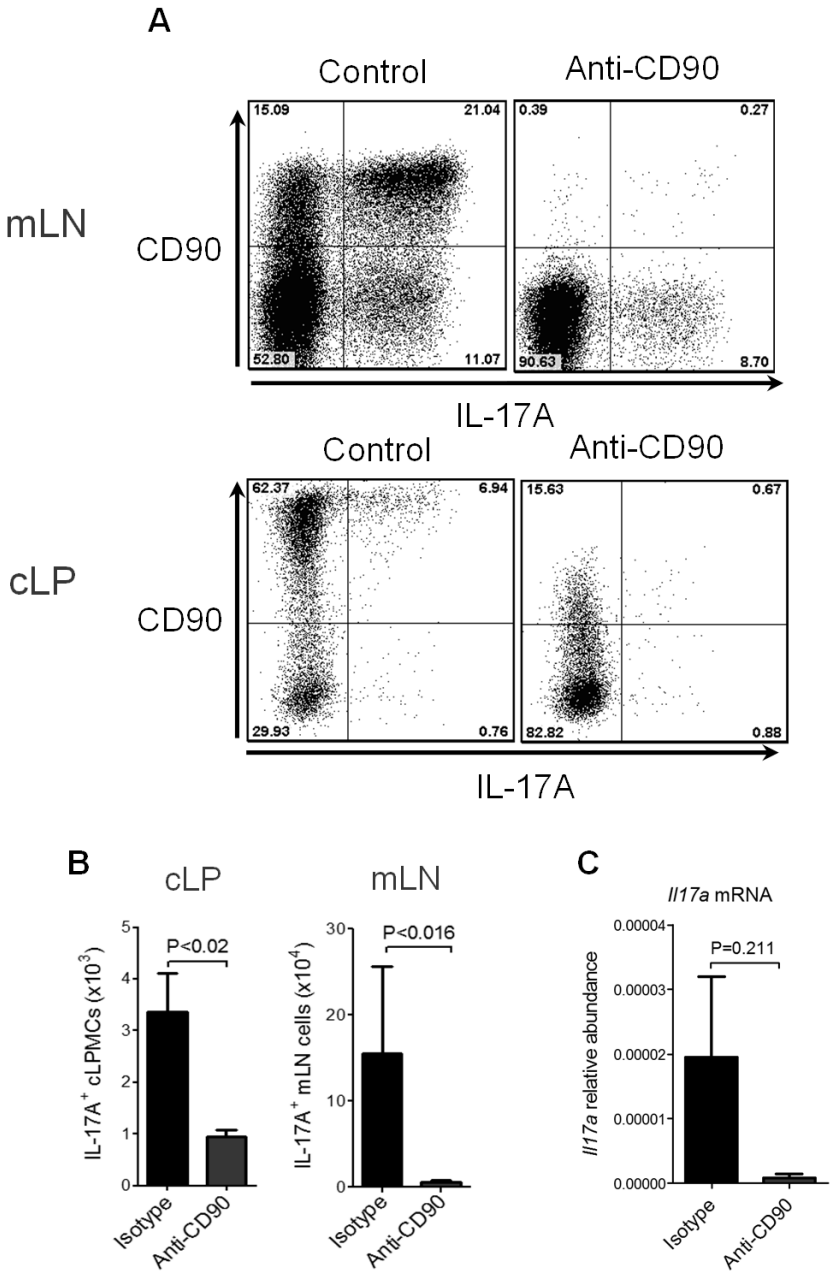

Figure 13. Anti-CD90 treatment depleted CD90 expressing cells. A Flow cytometry plot of cells from the mLN and cLP from TRUC mice following *in vivo* administration of anti-CD90 or control (isotype-matched) mAb. Cells were first stimulated with PMA and ionomycin. B Absolute number of IL-17A$^+$ cells in the cLP and mLN of TRUC mice after treatment with anti-CD90 or control isotype (after stimulation of cells with PMA and ionomycin). Bars represent mean cell number. Error bars indicate SEM. C qPCR quantifying *Il17a* mRNA in the colon of TRUC mice after treatment with anti-CD90 (n=4) or isotype (n=5). Bars indicate mean relative abundance of *Il17a* transcripts and error bars indicate SEM. Mice in this experiment were aged between 12-14 weeks old. Adapted from Powell *et al*., 2012.

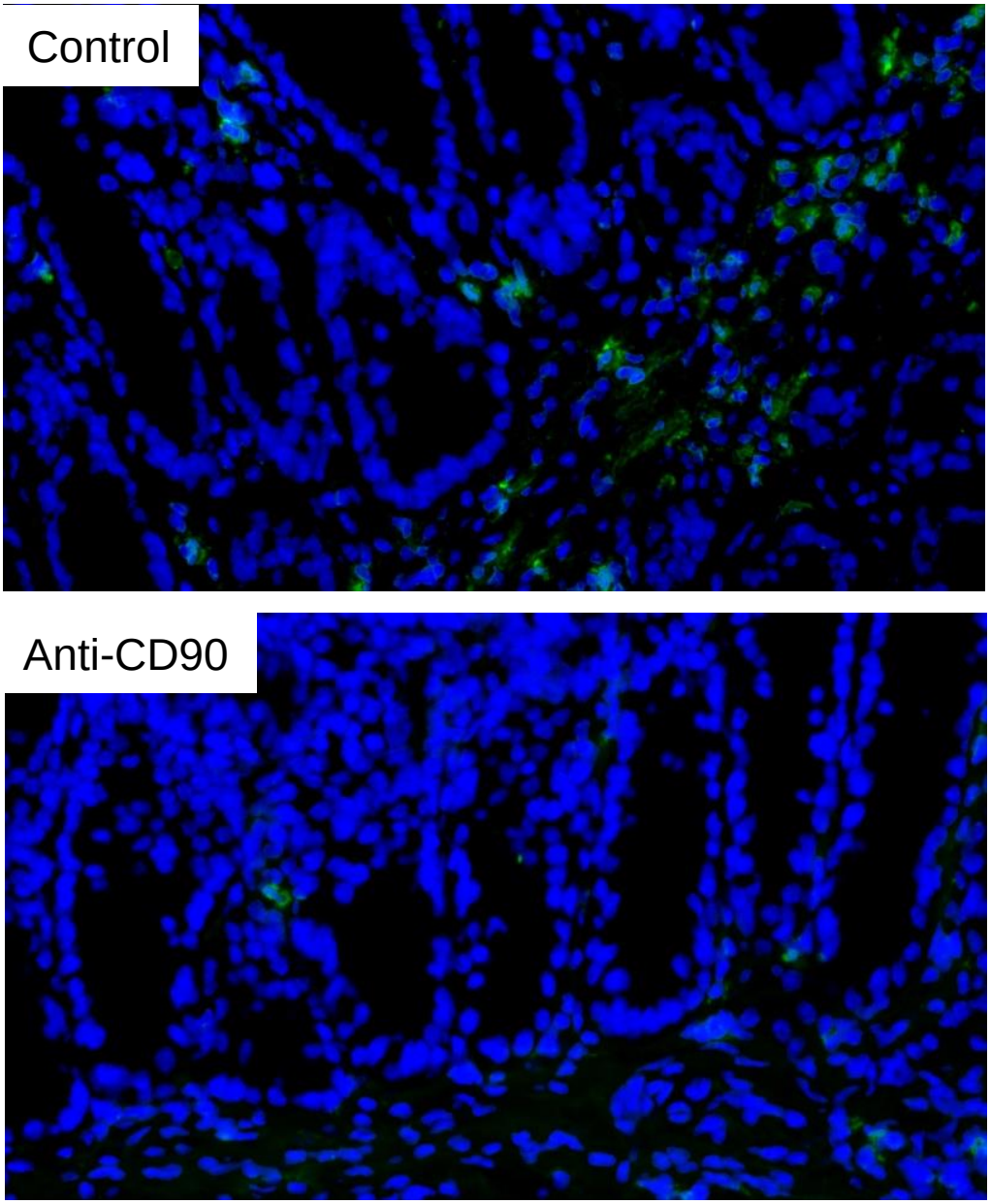

Figure 14. Anti-CD90 treatment depleted CD90 expressing cells. Immunofluorescence staining of colon segments harvested from TRUC mice following *in vivo* administration of anti-CD90 or control (isotype-matched) mAb. Sections were stained with anti-CD90-FITC. Nuclei were counter stained with DAPI. Magnification is the same in both panels (initially approximately x40, and further increased in equal proportion to generate the full page Figure). Adapted from Powell *et al*., 2012.

3.7 Depletion of CD90$^+$ cells significantly abrogated chronic TRUC disease

The impact of antibody-mediated depletion of CD90 expressing cells on TRUC disease parameters was also investigated. In these experiments the functional impact of CD4 depletion was also simultaneously investigated (the rationale and results for this intervention are presented in 3.10). TRUC mice treated with control antibody developed severe histological features of colitis, including marked epithelial hyperplasia, widespread crypt abscess formation, goblet cell depletion and infiltration of the cLP with mononuclear cells and PMNs (Figure 15A). In contrast, anti-CD90 treated mice had markedly improved histological appearances. The colitis scores in control antibody treated mice gave a median score of 12 (IQR 7-12), while anti-CD90 treated mice had a median histology score of 1 (0.25-1.75, $P<0.02$). Although the results of CD4 depletion are presented in section 3.10, it is noteworthy that mice treated with anti-CD90 mAbs had significantly less severe colitis than anti-CD4 treated mice, which did not differ significantly from control antibody treated mice (Figure 15A and B).

Other TRUC disease parameters were also changed by anti-CD90 treatment. Splenomegaly is a common feature of chronic TRUC disease (Figure 4E). In anti-CD90 treated mice there was a significant reduction in spleen size (median = 50mg, IQR 50-53.75mg) in comparison with control antibody treated mice (median = 90mg, IQR 82.5-105mg, $P<0.018$, Figure 16A). Colon weight was also significantly reduced in anti-CD90 treated mice (median = 315mg, IQR 225-337.5mg) in comparison with control antibody treated mice (median = 390mg, IQR 350-450mg $P<0.02$, Figure 16B). There was a tendency for anti-CD90 treated mice to exhibit improved weight gain in comparison with control mice over the course of the 5 week experiment. Mice treated with anti-CD90 had a median weight gain over the 5 week experiment of 6.4% (IQR 4-8.5%) in comparison with control antibody treated mice that only gained 1.7% (IQR -1.75-9.4%), however, this did not achieve statistical significance ($P<0.19$) (Figure 16C). Taken together these data are consistent with a key functional role for CD90$^+$ ILCs in TRUC IBD with a statistically significant difference seen in major disease parameters in mice treated with an anti-CD90 mAb, which was observed to significantly deplete the majority of CD90$^+$ cells in the colon

of TRUC mice. Notably, mice treated with anti-CD90 also had significantly less severe disease parameters than anti-CD4 treated mice (Figure 16 A-C).

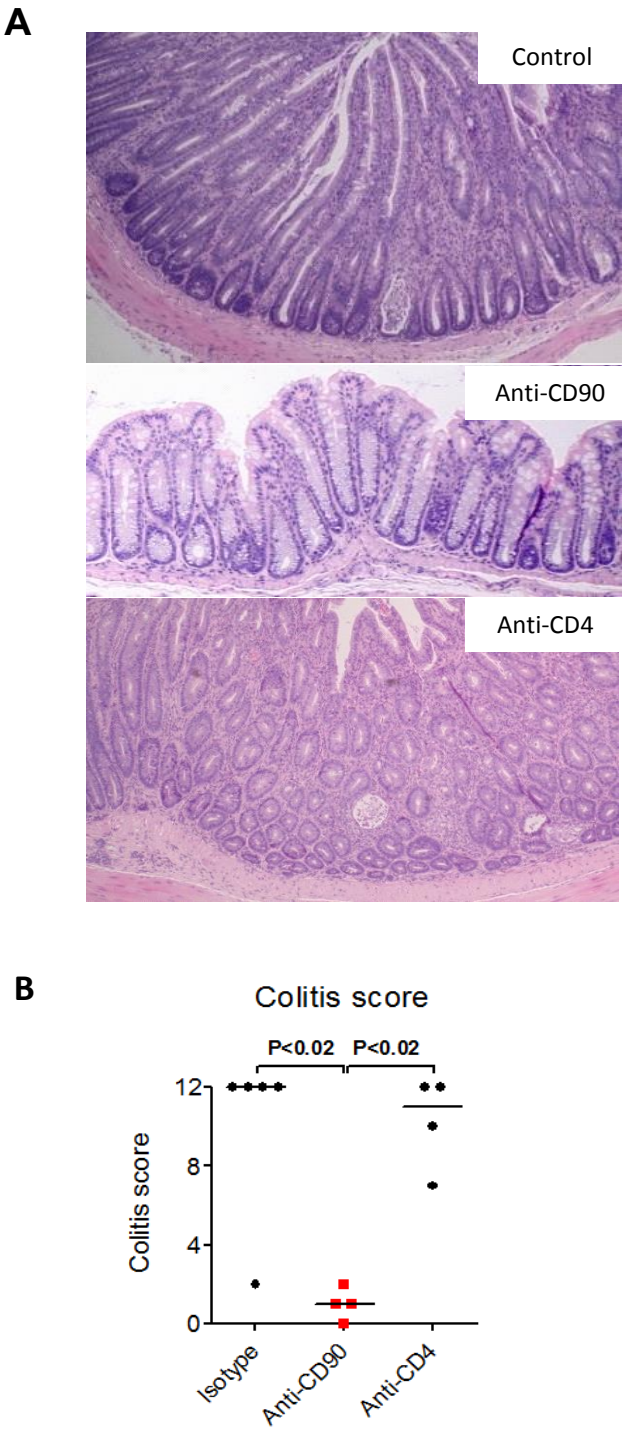

Figure 15. Anti-CD90 treatment significantly attenuated chronic colitis in TRUC mice, but anti-CD4 treatment was ineffective. A H&E stain showing the histological appearance of the distal colon of TRUC mice following treatment with anti-CD90, anti-CD4 or control (isotype-matched) mAb. Magnification is the same in both panels (approximately x40). Crypt abscess formation was readily visible in control mAb and anti-CD4 treated mice as depicted. B Colitis histology scores in the same study animals. The line represents the median colitis score. Each dot represents an individual animal. These data are all from a single experiment, which was not repeated. Adapted from Powell *et al.*, 2012.

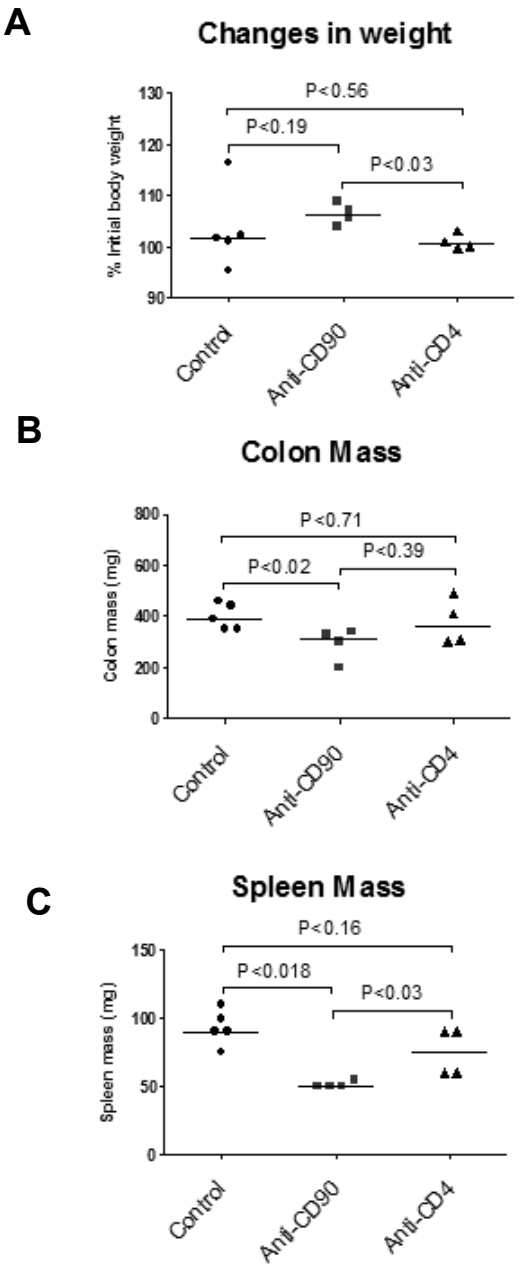

Figure 16. Anti-CD90 treatment significantly attenuated other disease parameters in TRUC mice, but anti-CD4 treatment was ineffective. A Change in body weight expressed as a % of initial body weight (final body weight/initial body weight x 100), B Colon mass and C spleen mass in TRUC mice treated with either control mAb (n=5), anti-CD90 (n=4) or anti-CD4 (n=4). These data are all from a single experiment, which was not repeated. Adapted from Powell *et al*., 2012.

3.8 There was a $CD4^+$ subset of $CD90^+$ ILCs in TRUC mice

A potentially important cell surface marker that distinguishes between different intestinal ILC subsets with apparently distinct functions is CD4. A population of $CD4^-$ $CD90^+$ ILCs have been described in the gut that appear to mediate colitis (Buonocore *et al*., 2010), whereas another population of $CD4^+$ $CD90^+$ ILCs may be involved in host resistance to intestinal pathogens (Sonnenberg *et al*., 2011). Therefore, the expression of CD4 by intestinal ILCs from TRUC mice was investigated.

Although CD90 expression was clearly seen in $CD45^+$ cLPLs from TRUC mice, there was very little CD4 expression (Figure 17A). In contrast, in the mLN of TRUC mice $CD4^+$ cells could be observed (Figure 17A). Notably, the majority of $CD4^+$ cells also expressed CD90 consistent with the likelihood that most $CD4^+$ cells present in the mLN of TRUC mice are $CD90^+$ $CD4^+$ double positive ILCs. On the other hand the majority of $CD90^+$ cells in the mLN of TRUC mice did not express CD4. These data indicate that there are (at least) 2 populations of $CD90^+$ cells in the mLN of TRUC mice characterised by differential expression of CD4.

The relative capacity of $CD4^+$ and $CD4^-$ cell populations to produce IL-17A was investigated using flow cytometry. In the colon there were very few $CD4^+$ cells and these cells did not express appreciable IL-17A (Figure 17B). Instead, most IL-17A production in the colon of TRUC mice was produced by $CD90^+$ cells. IL-17A production by CD90 and CD4 expressing cells was also investigated in the mLN of TRUC mice. IL-17A was produced by both $CD4^+$ and $CD4^-$ cell populations (Figure 17B). Interestingly, subdivision of mLN $CD90^+$ cells into $CD4^+$ double positive cells and $CD90^+$ $CD4^-$ (single positive) populations demonstrated that many $CD90^+$ $CD4^+$ cells and $CD90^+$ $CD4^-$ cell populations expressed IL-17A (Fig 18A). Although both single and double positive cells were frequently $IL\text{-}17A^+$, it was also noted that a significantly higher proportion of $CD90^+$ $CD4^+$ double positive cells expressed IL-17A in comparison with $CD90^+$ $CD4^-$ cells $P<0.019$, Figure 18B).

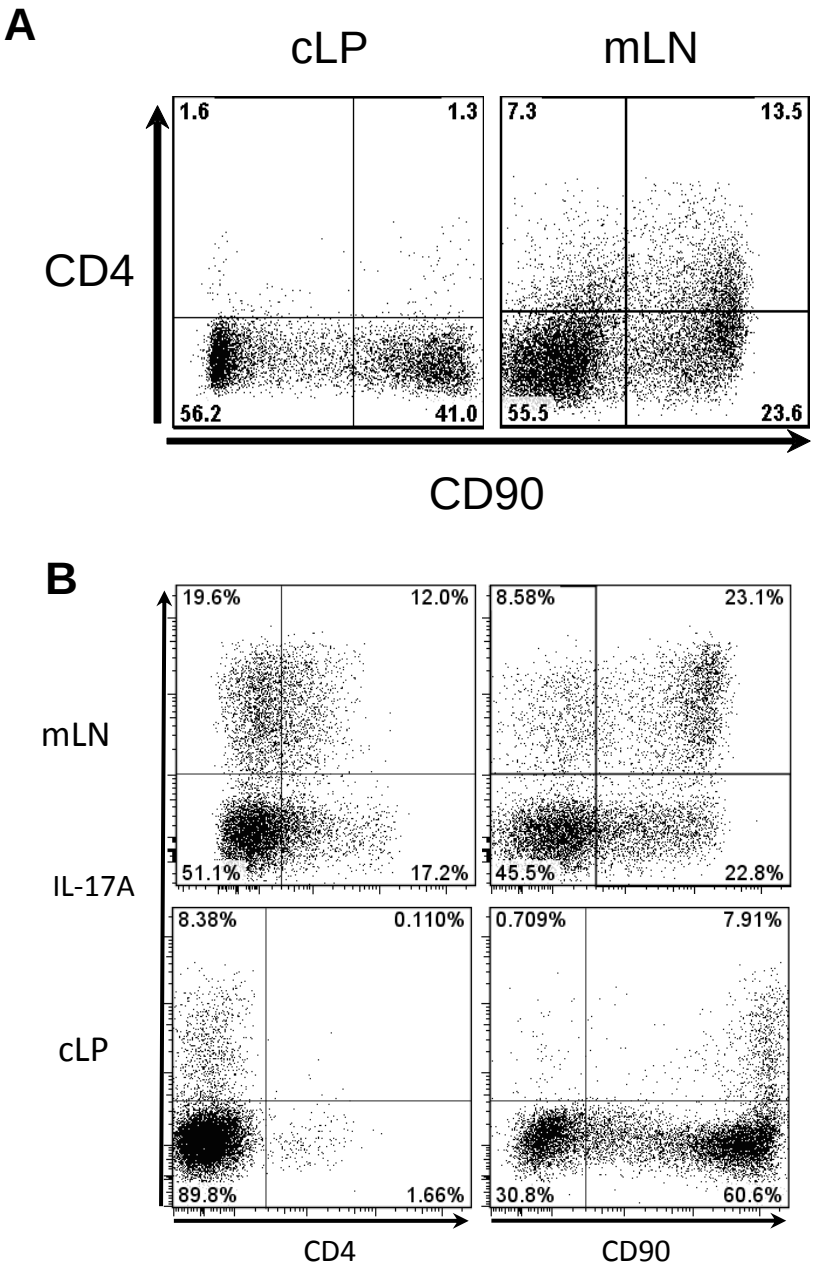

Figure 17. CD4 and CD90 expressing cells in TRUC mice. A CD4 and CD90 expression in the cLP and mLN of TRUC mice. B IL-17A expression in CD4 and CD90 cells in TRUC mice. Unfractionated cells were stimulated with PMA and ionomycin in the presence of monensin. Positive staining was defined by comparing unstimulated cells incubated with monensin alone. Cells were gated on live cells (and additionally with CD45 for cLP). The CD4 gate was defined by comparing analogous samples from mice receiving depleting anti-CD4 mAb (section 3.9). Data are representative of >4 individual experiments. Mice in this experiment were aged 12-14 weeks old. Adapted from Powell *et al*., 2012.

3.9 Depletion of $CD4^+$ cells failed to limit innate IL-17A expressing cells in TRUC mice

To establish the relevance of $CD4^+$ cells in TRUC disease, a depleting mAb was administered to TRUC mice. This experiment was performed at the same time as the depleting CD90 experiment in a parallel protocol using the same set of control antibody treated mice as comparison. Utilisation of a common control population of isotype antibody treated mice was enabled since the anti-CD90 and anti-CD4 mAbs are both rat antibodies of the same immunoglobulin subclass (IgG2b). Therefore, a common isotype control mAb clone was used for control mice (clone LTF-2). A common administration protocol was also used (Figure 12). One mg of anti-CD4 (clone GK1.5), or control antibody (clone LTF-2) was administered *i.p.* to 12-14 week old TRUC mice once weekly for 4 weeks. This clone has been shown to successfully deplete $CD4^+$ ILCs (Sonnenberg *et al.*, 2011). At the end of the experiment (48 hours after the final administration of antibody) mice were culled and organs harvested.

IL-17A production by $CD90^+$ $CD4^+$ double positive and $CD90^+$ $CD4^-$ single positive ILC populations was analysed by flow cytometry following administration of depleting anti-CD4 mAb or control antibody. In control mAb treated mice there was a population of $CD4^+$ and $CD4^-$ cells in the mLN that were producers of IL-17A (Figure 18C). However, following administration of anti-CD4, the $CD4^+$ population of cells was almost completely depleted (Figure 18C). However, there was still substantial production of IL-17A cells which were $CD90^+$ (Figure 18C). These data indicate that whilst anti-CD4 mAbs can be used to successfully deplete CD4 expressing cells, there is still substantial IL-17A production in anti-CD4 treated mice in the $CD90^+$ $CD4^-$ population.

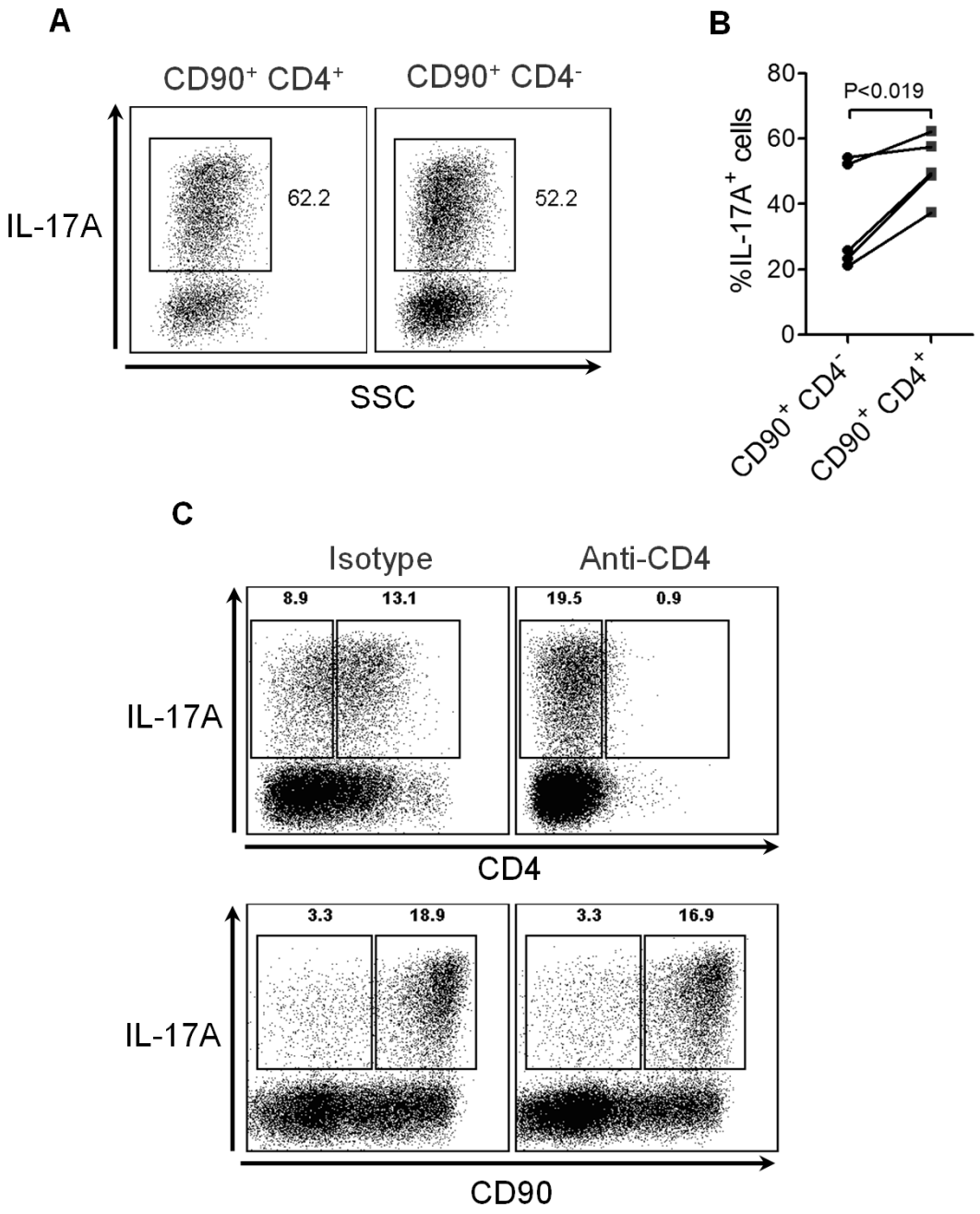

Figure 18. CD4$^+$ ILCs in TRUC mice produced IL-17A, but *in vivo* depletion of CD4$^+$ cells failed to limit innate IL-17A production. A Flow cytometry plot showing intracellular IL-17A expression in CD90$^+$ CD4$^+$ ILCs and CD90$^+$ CD4$^-$ ILCs in mLN of TRUC mice. Statistical analysis of biological replicates is shown in B. Each connected pair of dots are from a single mouse. C Flow cytometry plot showing CD90, CD4 and IL-17A expression in mLN of TRUC mice following *in vivo* treatment with an anti-CD4 or control (isotype) antibody. Cells were stimulated (in both A and C) with PMA and ionomycin (in the presence of monensin as previously described). Gating strategy has been described previously.

3.10 Antibody-mediated depletion of CD4 expressing cells *in vivo* failed to attenuate disease in TRUC mice

The functional significance of $CD4^+$cell depletion on TRUC disease parameters was investigated. Anti-CD4 treated mice developed severe colitis with pronounced epithelial hyperplasia, infiltration of the colon with polymorphonuclear cells and mononuclear cells, and crypt abscess formation (Figure 15A). There was no significant difference in histology scores in anti-CD4 or control antibody treated TRUC mice (Figure 15B). Other disease parameters were also assessed. There were no significant differences in body weight changes (Figure 16A), colon weight (Figure 16B) or spleen weight (Figure 16C) in control mAb or anti-CD4 treated mice.

3.11 Chronic TRUC IBD was IL-17A dependent

In view of the dominant IL-17A response observed in chronic TRUC IBD in organ culture, flow cytometry of $CD45^+$ immune cells, and by qPCR in TRUC mice, the functional significance of this cytokine was investigated. A neutralising anti-IL-17A (clone 17F3) or isotype control antibody (mouse IgG1) was administered *i.p.* every 3-5 days (7 doses in total) to TRUC mice aged 12 weeks old, when chronic colitis is well established (Figure 19). Seven mice received the neutralizing antibody and 8 mice received the control isotype matched antibody. Organs were harvested 48 hours after the final injection of anti-IL-17A or control antibody.

Administration of anti-IL-17A mAb significantly improved colitis scores ($P<0.02$) (Figure 20A and 20B). Indeed, 71% (5/7) of anti-IL-17A treated mice had complete resolution of colitis (colitis score=0). Improvements in histology sub-scores for epithelial hyperplasia and epithelial injury were particularly striking, with all 7 mice in the anti-IL-17A treated group scoring 0 for epithelial injury and 6/7 mice scoring 0 for epithelial hyperplasia. In this experiment IL-17A blockade did not significantly impact on other clinical features, such as colon weight (Figure 20C) or splenomegaly (Figure 20D).

Consistent with the biological activities of IL-17A, antibody blockade also markedly reduced the accumulation of $F4/80^-$ $CD11b^+$ $Gr\text{-}1^{high}$ granulocytes infiltrating the colon (Figure 21A and 21B). However, the proportion of cLP $F4/80^+$ macrophages was comparable in treated and untreated mice. Notably, there was also a small, but statistically significant proportional increase in the percentage of $CD90^+$ cells in the cLP of TRUC mice following IL-17A blockade. It is probable that this apparent increase is due to the marked reduction in granulocytes in anti-IL17A treated animals resulting in a relative increase in the number of $CD90^+$ cells. These data highlight one of the drawbacks of only measuring the relative proportion of cells in a tissue rather than quantifying absolute cell numbers.

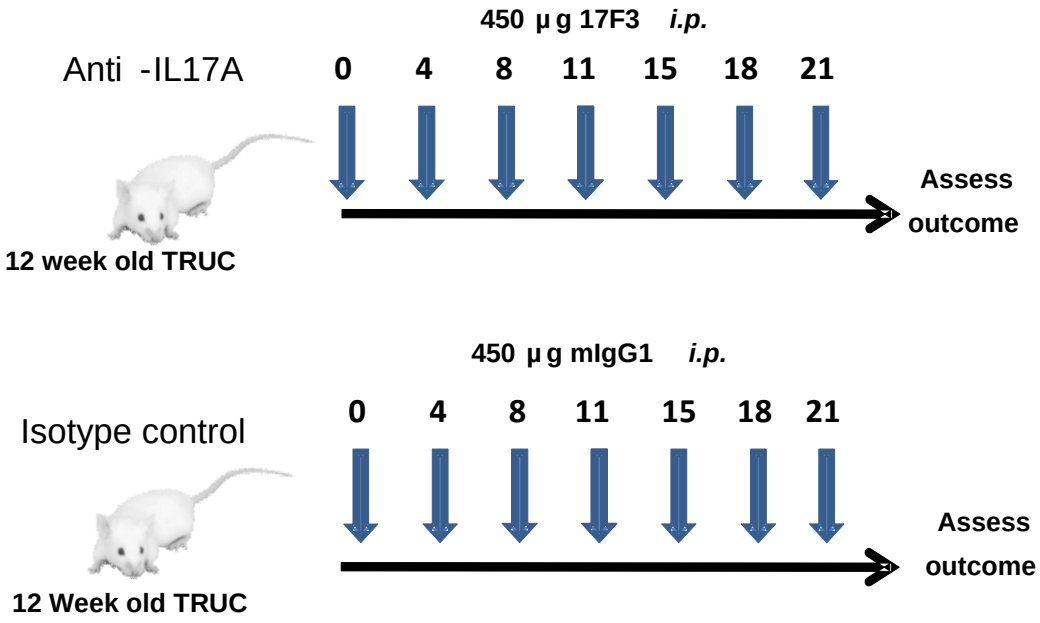

Figure 19. Experimental protocol followed to evaluate the functional role of Il-17A in chronic TRUC disease. 450µg of an anti-IL-17A (clone 17F3) or control isotype antibody (clone MOPC-21) was administered *i.p.* to 12 week old TRUC mice. Organs were harvested 2 days following administration of the final antibody dose. The anti-IL-17A neutralizing protocol was based on previously published data of blocking this cytokine *in vivo* in innate models of colitis (Buonocore *et al*., 2010). In this previously published study 375µg of anti-IL-17A was used twice per week, although it was a different clone.

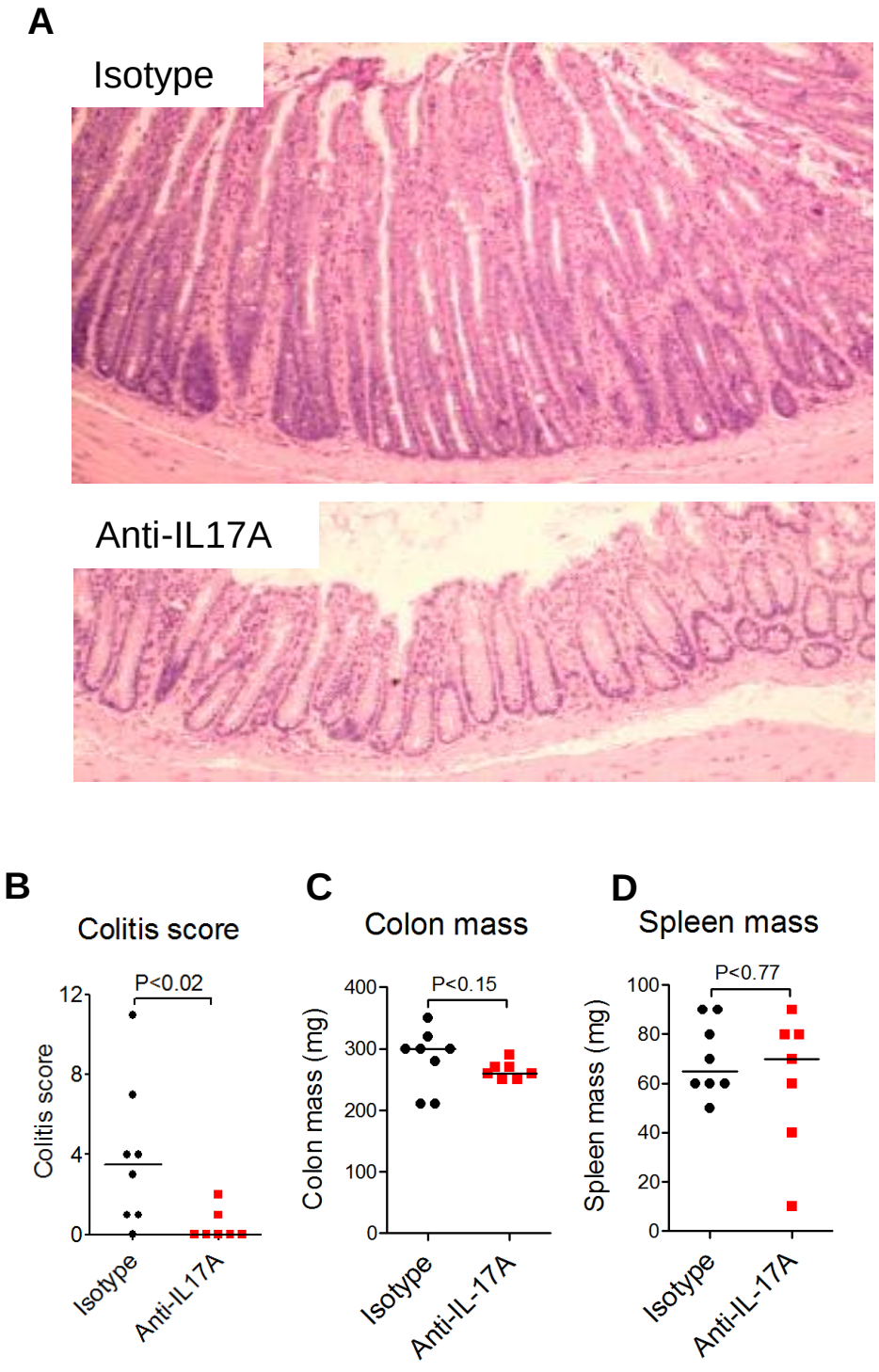

Figure 20. IL-17A neutralisation significantly attenuated TRUC colitis. A Histological appearance of the colon (H&E staining) of 12 week old TRUC mice receiving control mAb or anti-IL-17A. Magnification is the same in both panels (approximately x40). B Colon histology scores in TRUC mice receiving either control (isotype) antibody (n=8) or anti-IL-17A (n=7). C Colon mass and D spleen mass in control mAb or anti-IL-17A mAb treated TRUC mice. In all cases individual dots represent an individual mouse. In graphs lines depict median score or mass. Adapted from Powell *et al*., 2012.

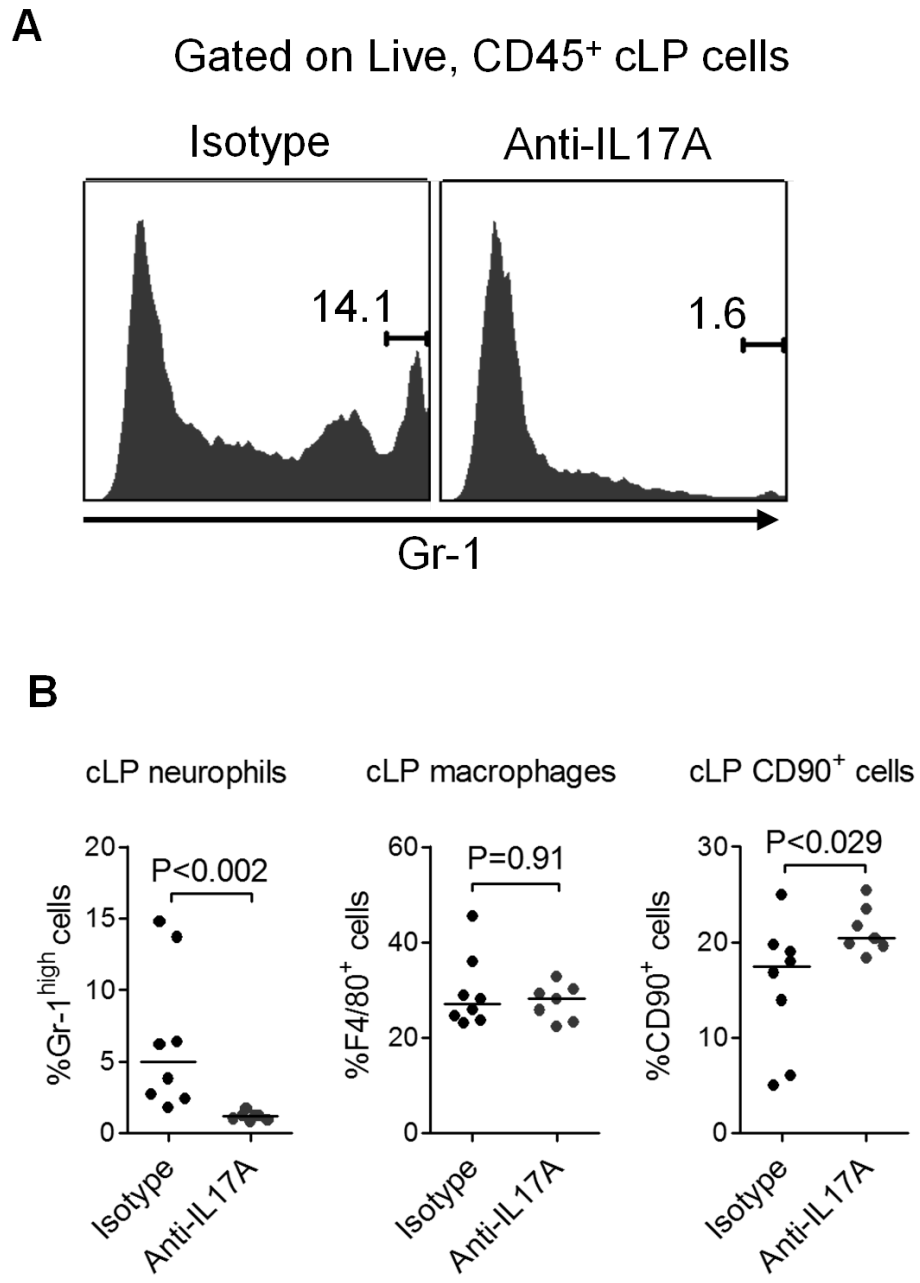

Figure 21. IL-17A neutralisation significantly reduced colonic neutrophil infiltration in TRUC mice. A Representative flow cytometry plot showing the proportion of Gr-1^{high} granulocytes in the colon of TRUC mice treated with either control (isotype) or anti-IL-17A mAbs. B Statistical analyses of the proportion of $CD11b^{+}$ $F4/80^{-}$ Gr-1^{high} neutrophils, $CD11b^{+}$ $F4/80^{+}$ macrophages and $CD90^{+}$ ILCs in the colon of TRUC mice treated with either control (isotype) antibody (n=8) or anti-IL-17A (n=7). Each dot represents an individual mouse and lines represent median % cells (as defined in y-axis) of CD45+ cells. Adapted from Powell *et al*., 2012.

3.12 Discussion

The results presented in the first results chapter shed new light on the TRUC model of IBD. For the first time it is demonstrated that IL-17A producing $CD90^{+}$ ILCs play an indispensable role in disease manifesting in TRUC mice. These novel data change the interpretation of the original papers discussing the TRUC model of IBD, as previously it was thought that this disease was entirely mediated by DCs producing TNFα (Garrett *et al*., 2007, Garrett *et al*., 2009). In comparison with *Rag2*$^{-/-}$ controls, IL-17A was increased in colon explant culture in TRUC mice. In agreement with these findings *Il17a* message was significantly increased in the colon of TRUC mice in comparison with *Rag2*$^{-/-}$ controls. Although statistically significant increased abundance of *Il17a* transcripts were detected in the colon of TRUC mice in comparison with *Rag2*$^{-/-}$ controls, it is noteworthy that *Il17a* mRNA levels were undetectable or at very low levels at the limit of detection in some TRUC mice. These data highlight the some of the limitations in using real time PCR on small (approximately 0.5cm) colon segments where RNA extracted from whole tissue, including epithelial cells and stromal cells are processed in the absence of exogenous stimulation. An alternative approach, to increase the probability of detecting appreciable changes in cytokine abundance, might be to purify cLPLs and stimulate them with powerful stimuli, such as with PMA and ionomycin prior to performing real time PCR experiments. Using greater cell numbers (e.g. using the entire cLPL population isolated) might also improve the sensitivity of quantitative PCR assays. For the first time the importance of IL-17A in chronic TRUC disease has been demonstrated. *In vivo* neutralization of IL-17A with a specific mAb was therapeutic in TRUC disease. It markedly reduced colitis histology scores and attenuated colonic infiltration with Gr-1^{high} granulocytes. These novel data show that IL-17A is functionally important in TRUC disease. Unlike other ILC-mediated models of colitis, which required simultaneous neutralization of IL-17A and IFNγ for optimal suppression of colitis (Buonocore *et al*., 2010), disease in TRUC mice could be abrogated by monotherapy with anti-IL-17A alone. Indeed, in TRUC mice IFNγ production was limited, consistent with the T-bet deficient status of TRUC mice, since T-bet has been shown to be a key transactivator of the IFNγ transcription (at least in T-cells) (Szabo *et al*., 2000). These data support the notion that excess IL-

17A production is pathogenic in the gut under appropriate conditions. These data are also consistent with host genetic factors playing a central role in host cytokine responses. Interestingly, despite the large number of genetic loci (163 genes) now associated with IBD (Jostins *et al*., 2012), variation at the *TBX21* locus has yet to be associated with this disease state. However, it has recently been shown that polymorphisms at the *TBX21* locus are associated with increased risk of ankylosing spondylitis, an autoimmune arthropathy (Cortes *et al*., 2013). Interestingly, ankylosing spondylitis is associated with subclinical or overt IBD in many patients and like IBD is also linked genetic variation in IL-23/IL17 axis components, such as *IL23R, IL12B, TYK2* and *IL6R* (Cortes *et al*., 2013).

In chronic TRUC disease the principle cell source of IL-17A was $CD90^+$ $NKp46^-$ $CCR6^+$ $ROR\gamma t^+$ $IL\text{-}7R^+$ cells which share phenotypic markers with ILC3 cells. Notably, there was also, albeit smaller, population of IL-17A producing cells in the $CD90^-$ population of cells in TRUC mice, most prominently seen in the mLN. Although the identity of these cells was not pursued in this thesis possible cellular sources include neutrophils (Ferretti *et al*., 2003) and NK cells (Hughes *et al*., 2009).

ILCs were functionally important in TRUC disease, since depletion with anti-CD90 monoclonal antibodies cells cured colitis. However, it should be noted that other non-ILC cell populations have also been shown to express CD90, including neurones (Feng *et al*., 2000), mesenchymal stem cells (Jones *et al*., 2002), and some other stromal cells (Owens *et al*., 2013). In many of the experiments performed at the beginning of this period of research there were some difficulties isolating cLPLs. A major problem encountered was a low yield of viable cells, which was particularly challenging in long experiments, which required cell stimulation following cell isolation. Defining some cell surface markers, such as CCR6, was technically difficult in stimulated cells, particularly cLPLs. Unlike the situation in T and B cell replete mice isolating immune cells from the colon of lymphopaenic mice is more challenging. For this reason many experiments were performed on mLN or spleen, since cells from these tissues can be readily isolated and analysed. Consequently, some of the data presented in this thesis should be interpreted bearing in mind which tissue is being presented in particular experiments/figures. This is a potentially important issue since it was observed that ILC populations appeared to differ in

different tissue compartments. For example, in the mLN some ILCs expressed CD4, whereas CD4 expressing ILCs were seldom seen in the colon, the primary site of disease in TRUC mice. Likewise, simultaneous staining for CD90 in the colon and mLN from the same donor mouse often showed CD90 expression to be seemingly higher in the colon in comparison with the mLN (Figure 10 and Figure 11). The role of the cell surface marker CD90 in ILCs is unknown and differential expression of CD90 in these different tissue compartments may reflect heterogeneity in ILCs from different tissues. Alternatively, the difference in CD90 expression in ILCs from the colon and mLN may reflect differences in the activation status of these cells. Indeed, CD90 modulates activation of other lymphoid lineages, such as $CD4^+$ T-cells (Bellio *et al*., 1991), and therefore, may also supply co-stimulatory/inhibitory signals to ILCs. Accordingly, it is possible that CD90 expression is differentially regulated at different tissue locations or disease states.

Although differential CD4 staining of ILCs in distinct tissue compartments in TRUC mice indicates that colonic ILCs and mLN ILCs are not interchangeable, the subsequent depletion studies performed in this study provide some useful mechanistic insights into the role of CD4 expressing ILCs in TRUC disease. CD4 expressing $CD90^+$ ILCs in TRUC mice were only seen in the mLNs and not in colon and even though $CD90^+$ $CD4^+$ ILCs are more potent producers of IL-17A than $CD90^+$ $CD4^-$ ILCs, depletion of CD4 expressing cells did not significantly impact disease outcome. Therefore, unlike the $CD4^+$ ILCs, which are responsible for IL-22 dependent resistance to *Citrobacter rodentium* infection (Sonnenberg *et al*., 2011), $CD4^+$ ILCs do not play a significant role in colitis development in TRUC mice. Indeed, even after depletion of CD4+ cells it could be seen that there was still marked production of IL-17A, indicating that $CD90^+$ $CD4^-$ ILCs can compensate for the absence of $CD4^+$ ILCs and cause disease.

A number of experimental/technical lessons were also learned during some of these early experiments, which may have some bearing on the results reported and may also inform refinement and improvement of future experiments. For instance, in some flow cytometry experiments the percentage of particular cell types was reported rather than absolute cell numbers. Although this approach may be useful in most settings a potential disadvantage of this approach is that relative changes in cell proportions may occur due to expansion or loss of an “unrecorded” or “unmeasured”

population of cells. In contrast, measuring absolute numbers of cells in particular tissues offers the advantage of being able to directly compare cell numbers across different animals and different experimental conditions. For instance, in depletion studies it may be more informative to detail the total number of cytokine producing cells in a particular tissue rather than the relative amount between different cells. The major reason for reporting relative proportions in this thesis was that whole colons were seldom used for flow cytometry experiments alone and instead additional pieces of colon were used to set up organ culture, RNA extraction, histology, etc. However, recognition of the merit of recording absolute cell numbers was realised and implemented in many of the later experiments.

Another potential problem was the use of the same clone of mAb for staining in flow cytometry experiments as the antibody clone employed to deplete cells *in vivo*. For example, the same clone of mAb used to deplete CD90 *in vivo* was also then used to detect the presence of CD90 expressing cells in subsequent flow cytometry experiments (in this case clone 30H12). It is possible that the staining mAb would be unable to recognise CD90 expressing cells since the epitope bound by the staining mAb would already be occupied by the depleting mAb which had previously been administered *in vivo*. However, since mAb binding to its specific Ag is a dynamic process the addition of the staining mAb in vast excess during the surface staining procedure would likely negate this potential issue. In any case, the number of IL-17$^+$ cells was markedly diminished after *in vivo* depletion, and since CD90$^+$ cells were the most prominent source of IL-17A in TRUC mice, these data provide indirect support for the assertion that this antibody depleted CD90 expressing cells *in vivo*. In future depleting experiments it may prove useful to use different antibody clones (or alternative markers, e.g. Sca-1 or IL-7R, see Figure 11) for surface staining to the ones used to deplete cells *in vivo*. Such an approach might improve confidence that lack of detection of a particular cell is really due to its loss/depletion rather than an inability to detect it experimentally for technical reasons.

In conclusion, data presented in this chapter demonstrate that disease in TRUC mice is dependent on IL-17A producing CD90$^+$ ILCs. These important data build on mounting evidence implicating overly exuberant immune activation as an important event in the development of chronic intestinal inflammation. Importantly, cytokine

producing ILCs have been identified in the gut of patients with Crohn's disease (Geremia *et al*., 2011) as well as in other preclinical models of IBD (Buonocore *et al*., 2010). Accordingly, ILCs should be added to the list of potentially important effector cells that may play an important role in the pathogenesis of IBD. Interestingly, ILCs such as those identified in TRUC mice in this thesis, as well as those reported by other groups, are linked to a variety of molecules in which genetic variation is associated with IBD. These include RORC, IL-23R, IL-12B, CCR6 and IL7R (Jostins *et al*., 2012). This raises the possibility that polymorphisms at these IBD associated loci may impact on the phenotype of ILCs in a functionally important way. This would raise the intriguing possibility that polymorphisms at genetic loci that impact on ILC phenotype have a critical bearing on IBD susceptibility. This would lead to a major paradigm shift in current thinking since it has always been assumed that increased disease susceptibility conferred by these IBD associated polymorphisms likely to result from their impact on the phenotype of mucosal T-cells rather than innate immune cells. Given the similarity of ILCs to T-cells in terms of cellular morphology, shared phenotypic markers, shared master transcriptional regulators and common cytokine secreting profiles it is of course possible that polymorphisms at genetic loci encoding proteins shared by T-cells and ILCs contribute to IBD risk. The relative contributions of ILCs and T-cells as effector cells in human IBD remains to be established. Although ILCs are numerically inferior to T-cells in the gut of IBD patients (Geremia *et al*., 2011) it is likely that activation of these cells is triggered by different stimuli. T-cells are primarily activated through their recombined antigen-specific T-cell receptor, with or without co-stimulation. By definition ILCs lack antigen-specific receptors and instead are likely to be stimulated by cytokines or other innate signals in the local environment. In view of the likelihood that the effector function of ILCs and T-cells are activated by distinct triggers implies that either the dynamics or environmental conditions which result in activation of these different cell types is likely to be different. It is tempting to speculate that ILCs are present at the mucosal barrier surfaces as rapid responders to changes in the local tissue cytokine milieu to enable immediate mobilisation of a potent effector response, irrespective of the offending pathogen. On the other hand T-cells are present to provide response specificity to previously encountered pathogenic organisms and build up immunological memory of commonly

encountered pathogens. Indeed, IL-22 producing ILCs are crucial in early resistance to *Citrobacter rodentium* infection (Sonnenberg *et al*., 2011), whereas enduring immunity is conferred by IL-22 producing $CD4^+$ T-cells (Basu *et al*., 2012). Therefore, mucosal ILCs and T-cells might contribute distinct but complimentary arms of the immune responses which operate in a co-ordinated manner to improve the effectiveness of host immunity. Unfortunately, it is possible that this system might become perturbed/dysregulated and result in chronic, immune mediated disease, such as IBD, particularly in individuals with genetic variation in molecules which impact on ILC and T-cell phenotype. In the next chapter of this thesis some of the proximal cytokine signals responsible for triggering effector function of ILCs were investigated.

CHAPTER 4

Results: Defining the proximal molecular signals responsible for driving activation of ILCs in TRUC mice

The first results chapter of this thesis has strongly implicated IL-17A producing $CD90^+$ $IL\text{-}7R^+$ ILCs in the colon of TRUC mice as functionally important drivers of chronic TRUC disease. To further understand the immune mechanisms responsible for driving innate immune mediated pathology in TRUC mice, it is also desirable to understand and define the proximal molecular signals responsible for driving activation of these cells *in vivo*. ILCs have only recently been identified as important mediators of intestinal inflammation and consequently there are relatively few data regarding the molecular basis for their activation.

In this chapter the proximal cytokine signals potentially responsible for driving ILC effector function in TRUC mice was investigated, focussing on cytokines that stimulate other ILC populations, such as IL-23, as well as cytokines which are thought to play an important role driving IL-17 responses in disease settings.

. IL-23 has been shown to play an important role in the innate immune mediated colitis model occurring when 129 SvEv *Rag1*$^{-/-}$ mice are inoculated with *Helicobacter hepaticus* (Hue *et al*., 2006) and has been shown to stimulate innate IL-17A production in other models of colitis (Buonocore *et al*., 2010). In addition to IL-23, a number of other molecules may be important for the differentiation and/or maintenance of Th17 cells, including IL-1β (Shaw *et al*., 2012), IL-6 (Hu *et al*., 2011) and TL1A (Pappu *et al*., 2008). Recently, it has already been shown that IL-1β triggers activation of intestinal ILCs (Coccia *et al*., 2012). IL-6 is a pleiotropic cytokine that may be important in IBD. Peripheral blood and colonic lamina propria leukocytes produce excess IL-6 in IBD (Reinecker *et al*., 1993; Suzuki *et al*., 1990), often at levels correlating with disease activity (Hyams *et al*., 1993). A polymorphism at the *IL6* locus is linked with early-onset CD (Sagiv-Friedgut *et al*.) and polymorphisms at loci encoding IL-6 receptor (IL-6R) signalling components, such as *STAT3*, *JAK2* and *TYK2* are associated with increased IBD risk (Jostins *et al*.,

2012). IL-6 plays a functionally important role in animal models of IBD, although the assumption has been made that the therapeutic mechanism of IL-6 blockade was attributable to limitation of T-cell mediated pathology (Atreya *et al*., 2000; Kitamura *et al*., 2004; Mitsuyama *et al*., 2006; Naito *et al*., 2004), since IL-6 contributes to intestinal Th17 differentiation (Hu *et al*., 2011). The role of IL-6 in innate immune mediated chronic intestinal pathology is unknown. In view of the perceived role of IL-6 in driving adaptive IL-17 production, the hypothesis that IL-6 would be a functionally important driver of innate IL-17 production in the gut in TRUC mice was tested.

Finally, there has been considerable interest in TL1A, a member of the tumour necrosis factor super family (also called TNFSF15) in IBD. SNPs at the locus encoding this protein confer an increased risk of IBD (Jostins *et al*., 2012). TL1A expression is increased in inflamed gut in IBD patients (Bamias *et al*., 2003), and has been shown to play an important functional role in animal models of intestinal inflammation (Meylan *et al*., 2011). Crucially, TL1A promotes $CD4^+$ T-cell effector function (Bamias *et al*., 2003), including Th17 responses (Pappu *et al*., 2008). The hypothesis that the cytokines IL-23, IL-6 and TL1A might also be important for innate IL-17A production, through activation of ILCs in TRUC mice was tested.

4.1 IL-23 stimulated innate IL-17A production in TRUC disease

To determine whether IL-23 induced IL-17 production by cells from TRUC mice, unfractionated cells from different tissues from TRUC mice were cultured in the presence or absence of recombinant IL-23 for 24 hours. The production of IL-17A by innate immune cells in these tissue compartments was then measured by ELISA. There was significant IL-17A protein induced by recombinant IL-23 in all tissues examined, including unfractionated colonic LPLs, mLN cells and splenocytes from TRUC mice (Figure 22). Notably, IL-17A production was most pronounced in the colon and draining lymph nodes compared to the spleen of TRUC mice.

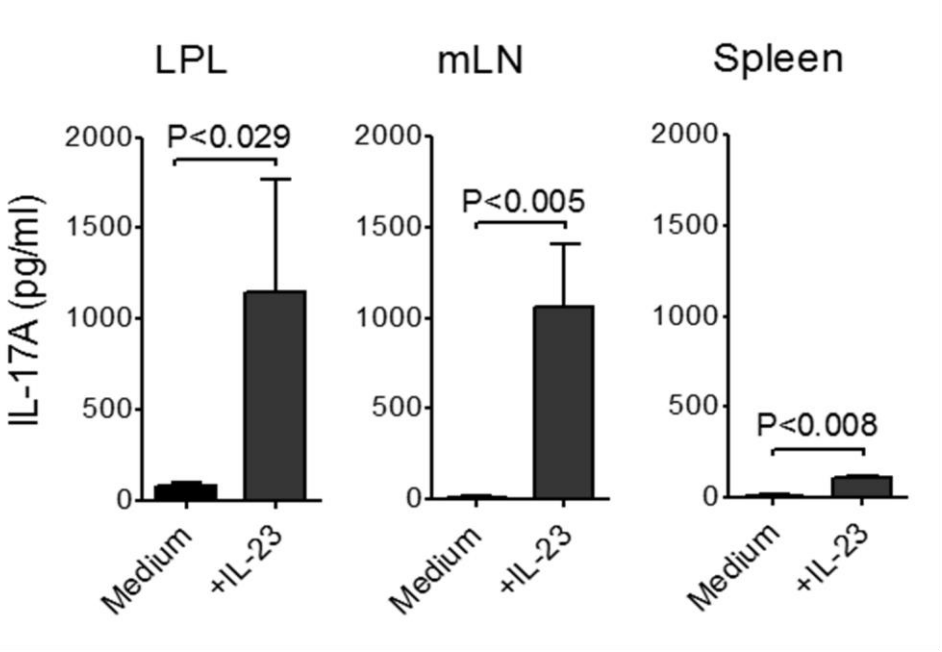

Figure 22.IL-23 stimulated innate IL-17A production in TRUC mice. Unfractionated cells from the cLP (1x 10^5/mL, n=4 individual mice/biological replicates for each experimental condition), mLN (1x 10^5/mL) (6 individual mice) or spleen (2x10^6/mL) (5 individual mice) were cultured in the presence of IL-23 (20ng/mL) or medium alone for 24 hours. IL-17A concentration was measured in culture supernatants by ELISA. Bars denote mean and error bars denote SEM of IL-17A production in culture supernatants of biological replicates. These data are a composite of 2 different experiments using identical experimental conditions. Adapted from Powell *et al*., 2012.

4.2 IL-23 stimulated CD90$^+$ NKp46$^-$ ILCs to produce IL-17A

In the previous chapter it was demonstrated that ILCs were the major source of IL-17A in TRUC mice, at least when cells were stimulated with PMA and ionomycin. Therefore, to determine whether IL-23 was responsible for triggering cytokine production by CD90$^+$ ILCs in TRUC mice, cells were stimulated with IL-23 followed by intracellular IL-17A staining. PMA and ionomycin stimulation was used as a positive control. Unstimulated cells treated with monensin alone were used as a negative control.

In these experiments unfractionated mLN cells were harvested from TRUC mice, since this tissue compartment is readily accessible and was a rich source of IL-17A producing ILCs in TRUC mice. Unfractionated mLN cells were cultured with medium alone (unstimulated), IL-23 (20ng/mL) or with PMA and ionomycin for 4 hours (last 2 hours with monensin added). Cells were then fixed (2% paraformaldehyde), stained with appropriate cytokine specific antibodies and then flow cytometry performed. As expected PMA and ionomycin stimulated marked production of IL-17A and to a lesser extent IL-22 in CD90$^+$ NKp46$^-$ ILCs from the mLN of TRUC mice (Figure 23). However, IL-23 also induced IL-17A and to a lesser extent IL-22 by ILCs from TRUC mice. IL-23 had little effect on IFNγ production by ILCs from the mLN of TRUC mice (Figure 23).

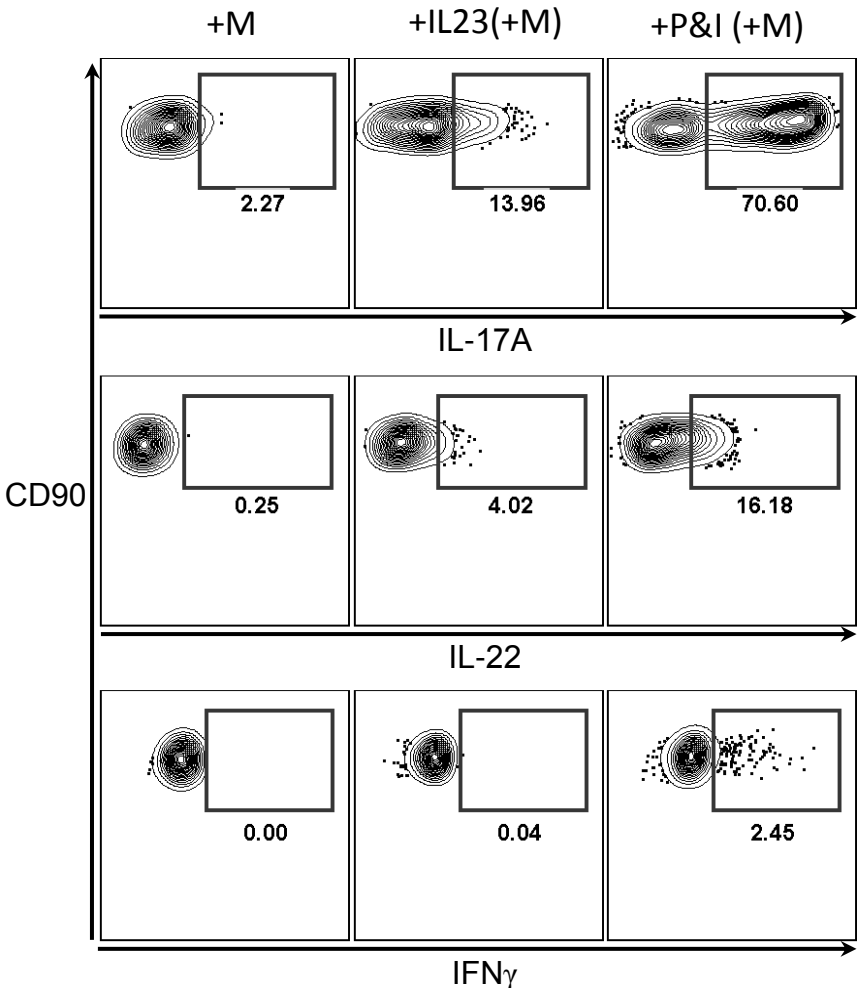

Figure 23. IL-23 stimulated IL-17A production by ILCs in TRUC mice – analysis by flow cytometry. Representative flow cytometry plots showing the proportion of cytokine expressing ILCs (gated on live, NKp46$^-$ CD90$^+$ cells) from mLN from TRUC mice following stimulation with IL-23 (20ng/mL), PMA and ionomycin (P&I, positive control) or unstimulated (Monensin only). Cells were stimulated for 4 hours (last 2 hours monensin was added). In the unstimulated condition monensin was added for the final 2 hours (+M). In this experiment mLN were pooled from 4 individual TRUC mice and plated out at 1x10^6 cells prior to stimulation. These data are representative of >3 individual experiments.

4.3 IL-23 mRNA expression in the colon of TRUC mice

In view of the ability of IL-23 to stimulate innate IL-17A production, the expression of transcripts encoding the different subunits of IL-23 was determined in TRUC and *Rag2*$^{-/-}$ mice. IL-23 is a heterodimer comprised of IL-12p40 (a subunit which it shares with IL-12) and the unique subunit IL-23p19, encoded by the *Il12a* and *Il23* genes respectively.

Accordingly, the abundance of *Il12a* and *Il23* transcripts was quantified in the colon of TRUC and *Rag2*$^{-/-}$ mice by qPCR. Both transcripts were present in the distal colon of TRUC and *Rag2*$^{-/-}$ mice (Figure 24). However, as well as being at low abundance, there was no statistically significant difference in the abundance of these transcripts between TRUC and *Rag2*$^{-/-}$ mice (Figure 24).

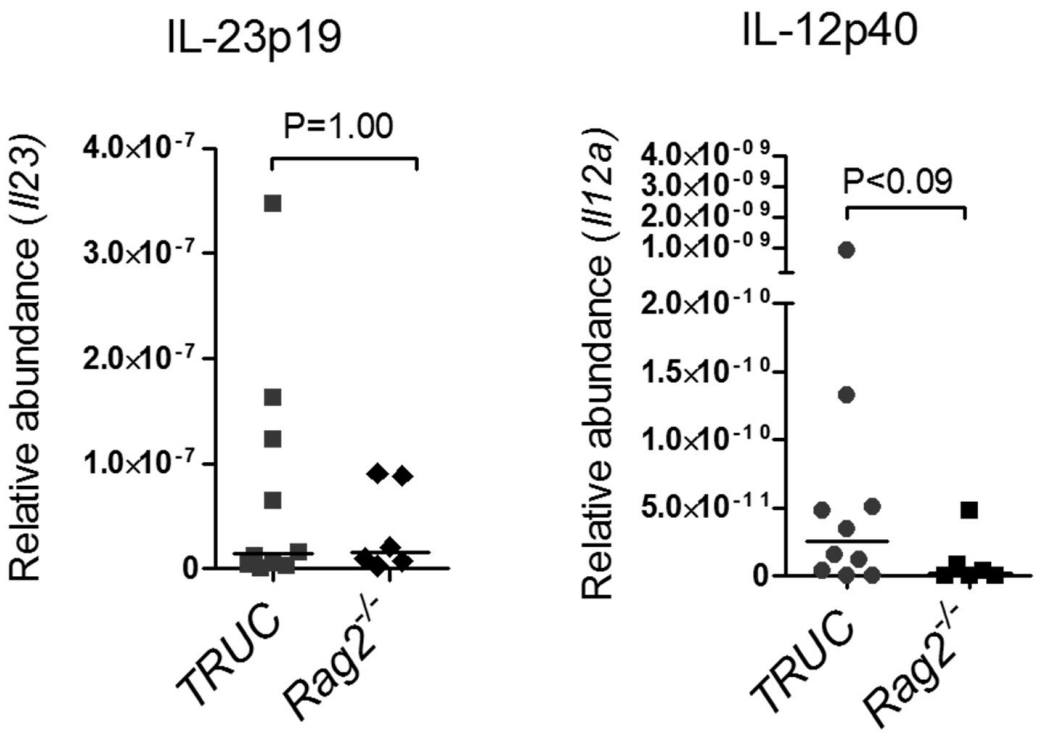

Figure 24. Transcripts encoding the IL-23 subunits *Il12a* (IL-12p40) and *Il23* (IL-23p19) were present in the colon of TRUC mice. qPCR analysis of transcripts encoding *Il12a* and *Il23* in distal colon of TRUC (n=10) and *Rag2*$^{-/-}$ (n=6) mice. Each dot represents an individual mouse. Line depicts median relative abundance of cytokine transcripts in the colon.

4.4 IL23p19 blockade attenuated colitis in TRUC mice

To determine the functional importance of IL-23 in TRUC disease, *in vivo* blocking experiments were performed with a neutralising antibody that only recognises the IL-23 specific subunit p19, and therefore blocks IL-23, but not IL-12. This mAb has been shown to neutralize IL-23 activity *in vivo* (Hagemann *et al*., 2008).

Anti-IL23p19 (n=4) or control isotype (n=7) antibodies were administered (*i.p.)* to TRUC mice aged 12 weeks old according to the protocol illustrated in Figure 25. Most notably, administration of anti-IL-23p19 significantly reduced chronic colitis in TRUC mice**.** The histological appearances of the distal colon of TRUC mice demonstrated marked reduction in colitis (Figure 26A) and significantly reduced histology scores in comparison with control antibody treated mice ($P<0.01$, Figure 26B). Anti-IL-23p19 treatment also significantly reduced colon weight in TRUC mice ($P<0.05$, Figure 26C). However, anti-IL-23p19 treatment had no impact on the splenomegaly in TRUC mice (Figure 26D). Together, these data indicate that neutralisation of IL-23 attenuates colonic disease in TRUC mice and demonstrate that IL-23 plays an important role in the innate immune mediated colitis present in TRUC mice.

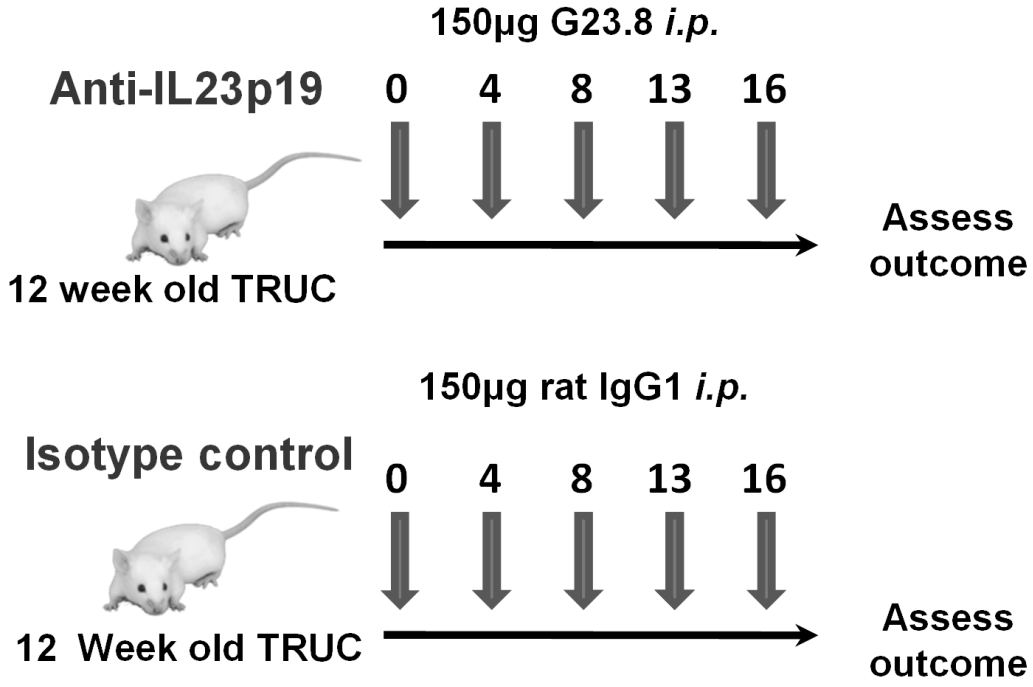

Figure 25. Experimental protocol followed to evaluate the functional role of IL23 in chronic TRUC disease. 150µg of anti-IL23p19 (clone G23.8) mAb (n=4) or control isotype antibody (n=7) was administered i.p. to 12 week old TRUC mice. Organs were harvested on day 19 of the experiment. Dosing regimen of anti-IL23p19 treatment was empirically based on the affordability of this expensive reagent. There are no data available regarding the use of this commercially available mAb in innate models of colitis. However, a neutralizing anti-IL23p19 mAb has previously been used *in vivo* in a mouse model of arthritis at 100µg every 6-7 days. In that experiment this dosage of mAb was calculated based on data from a functional assay (Cornelissen *et al*., 2013). Similarly, 100µg of G23.8 administered twice weekly has been shown to be biologically active (Hagemann *et al*., 2008).

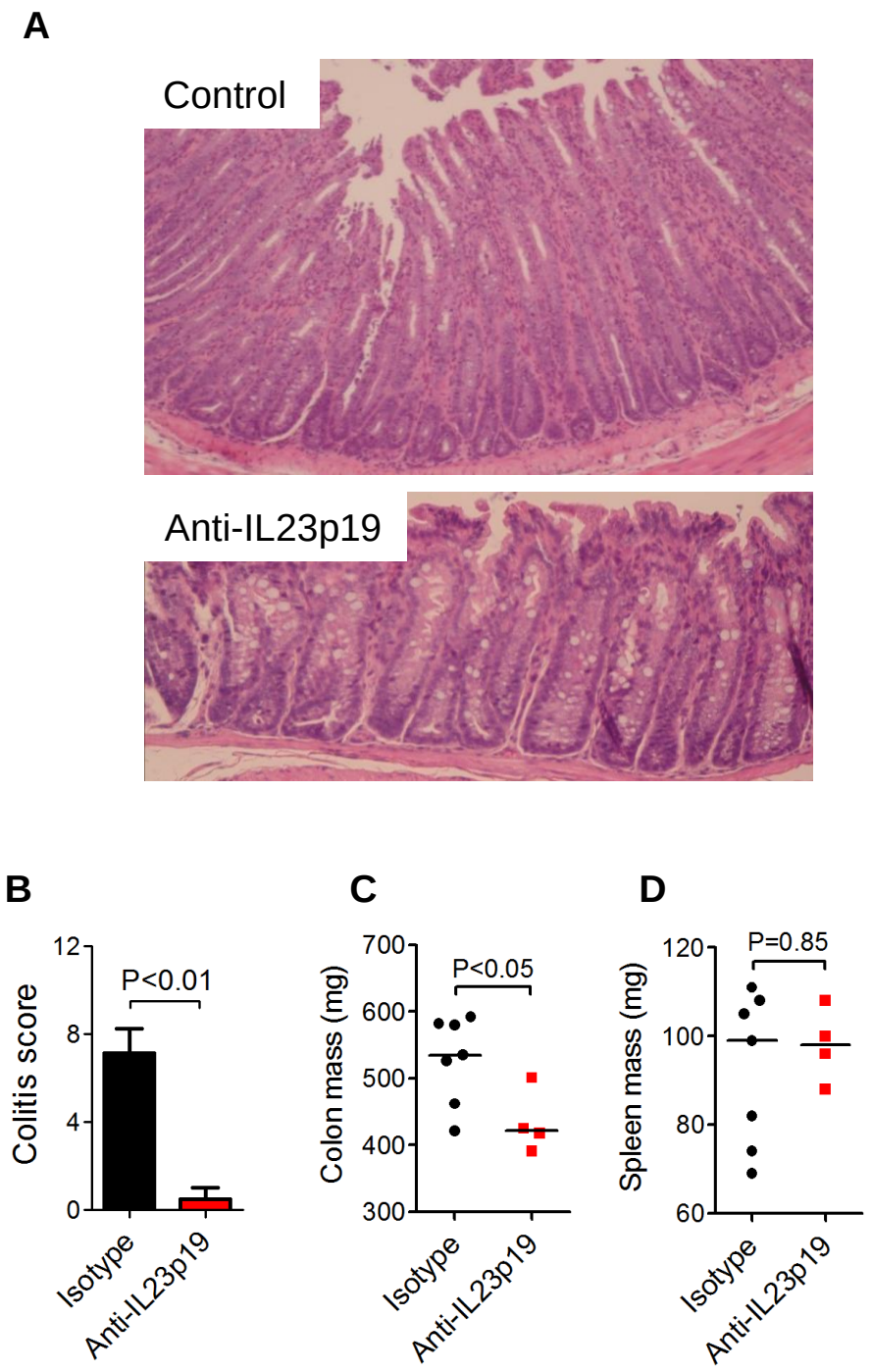

Figure 26. IL-23p19 blockade significantly reduced colitis in TRUC mice. A Representative H&E staining of distal colon specimens from TRUC mice treated with anti-IL23p19 (n=4) or control antibody (n=7). Magnification is the same in both panels (approximately x40). B Colitis histology scores, C colon mass and D spleen mass, following treatment with anti-IL23p19 (n=4) or control antibody (n=7). In Figure B the bars represent mean colitis score and error bars denote SEM. In Figures C and D each dot represents an individual mouse. Line depicts median mass. Adapted from Powell *et al*., 2012.

4.5 IL23p19 blockade reduced innate IL-17A production in TRUC mice

Since IL-23 stimulated innate IL-17A production in TRUC mice and plays a functionally important role in TRUC disease, it would be expected that IL-23 blockade would reduce colonic expression of IL-17A. IL-17A production in explant colon cultures was measured in TRUC mice treated with anti-IL-23p19 or control mAb. Importantly, *in vivo* IL-23 blockade significantly reduced spontaneous IL-17A production in explant organ culture ($P<0.035$, Figure 27A).

Using qPCR, mRNA encoding IL-17A was also quantified in the colon of TRUC mice following treatment with anti-IL-23p19 or control mAb. Following treatment, a 0.5cm segment of distal colon was immediately snap frozen in Trizol reagent, pending RNA extraction, cDNA synthesis and qPCR using IL-17A specific PCR primers. qPCR demonstrated that *Il17a* mRNA was reduced in the colon of anti-IL23p19 treated mice in comparison to control mAb treated mice, although this did not achieve statistical significance ($P<0.153$, Figure 27B).

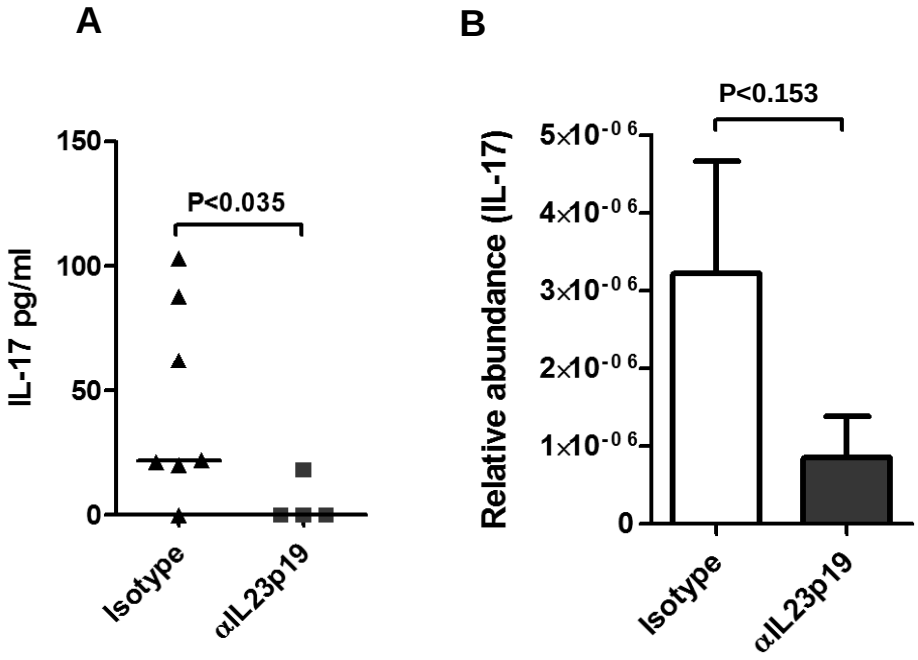

Figure 27. IL-23p19 blockade reduced colonic IL-17A expression in TRUC mice. A Explant organ culture from TRUC mice treated with anti-IL23p19 (n=4) or control antibody (n=7). Each dot represents an individual mouse. Line depicts median . B From the same mice transcripts encoding *Il17a* in the distal colon were quantified by qPCR. Bars represent mean relative abundance of *Il17*a mRNA and error bars denote SEM. Blockade experiments were performed in a single experiment and not repeated. Adapted from Powell *et al*., 2012)

4.6 IL-6 expression was increased in TRUC mice in comparison with *Rag2*$^{-/-}$ controls

In view of the prominent IL-17 response observed in TRUC colitis, and the perceived role of IL-6 in driving IL-17A responses (at least in the adaptive immune system), the role of IL-6 in innate IL-17A responses in the gut of TRUC disease was examined. The concentration of IL-6 in the serum of TRUC mice and age matched control *Rag2*$^{-/-}$ mice was measured by ELISA. Serum levels of IL-6 were significantly increased in TRUC mice in comparison with *Rag2*$^{-/-}$ controls ($P<0.0025$, Figure 28A). Notably, IL-6 was undetectable in the serum of *Rag2*$^{-/-}$ mice without intestinal inflammation. In contrast, IL-6 was detectable and significantly elevated in all TRUC mice.

The concentration of IL-6 in culture supernatants following culture of colon explants was measured by ELISA. In contrast to the serum, IL-6 was detectable in organ culture from TRUC and *Rag2*$^{-/-}$ mice. However, IL-6 levels were significantly increased in explant cultures from TRUC mice in comparison with *Rag2*$^{-/-}$ controls ($P<0.0028$, Figure 28B).

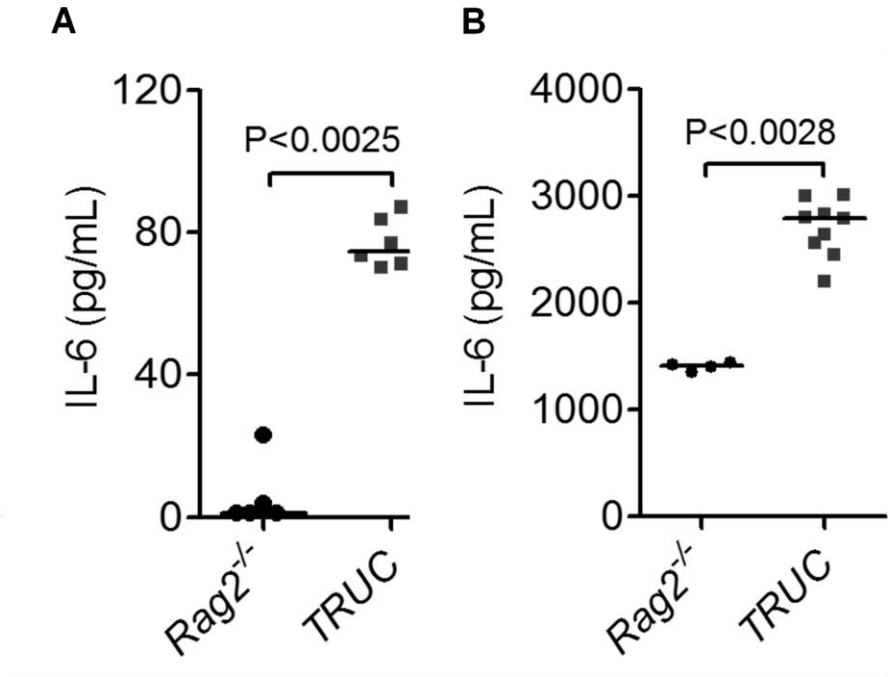

Figure 28. Increased IL-6 in TRUC disease. A The concentration of IL-6 in the serum of 12 week old TRUC (n=6) and *Rag2*$^{-/-}$ (n=5) mice, measured by ELISA. B Concentration of IL-6 in culture supernatants of colon explants from TRUC (n=9) and *Rag2*$^{-/-}$ (n=4) control mice. Each dot represents an individual mouse. Lines depicts median

4.7 ILCs from TRUC mice expressed the IL-6R

IL-6R expression by ILCs from TRUC mice was investigated by flow cytometry. Splenocytes were stained with NKp46, CD90 and IL-7R to identify ILCs ($NKp46^-$ $CD90^+$ $IL\text{-}7R^+$). By gating on the ILC population, the relative staining of IL-6R on ILCs was determined. In contrast to the lack of specific staining with the control isotype antibody, there was marked positive staining using the specific anti-IL-6R antibody (Figure 29A).

IL-6R expression was also analysed in ILCs from the colon of TRUC mice. IL-6R expression in colonic ILCs were identified in the same way as in the spleen ($NKp46^-$ $CD90^+$ $IL\text{-}7R^+$). As in the spleen, there was a lack of specific staining with the control isotype antibody, but marked positive staining using the specific anti-IL-6R antibody (Figure 29B).

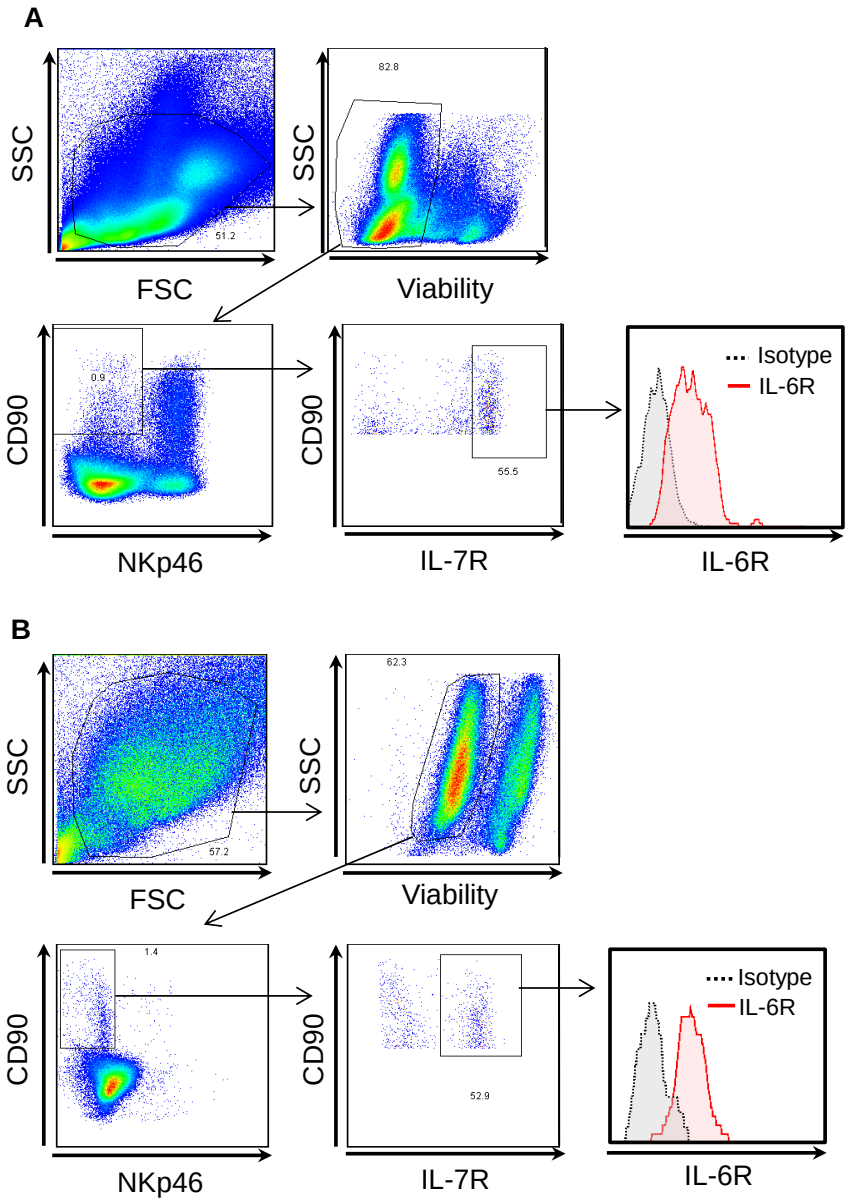

Figure 29. Splenic and colonic ILCs from TRUC mice expressed IL-6R. IL-6R (red line) or isotype (black dotted line) staining of A $CD45^+$ $NKp46^-$ $CD90^+$ $IL\text{-}7R^+$ ILCs from the spleen of TRUC mice and B $NKp46^-$ $CD90^+$ $IL\text{-}7R^+$ ILCs from the colon of TRUC mice.

4.8 IL-6 stimulated innate IL-17A production in TRUC mice

Since IL-6 it thought to play an important role in shaping adaptive IL-17 responses, the role of IL-6 in innate IL-17A responses in TRUC mice was investigated. In the previous Chapter it was demonstrated that unfractionated mLN cells are a particularly rich source of innate IL-17A production in TRUC mice (Figure 7). Therefore, unfractionated mLN cells from TRUC mice were cultured in the presence of IL-6 (50ng/mL) or with medium alone. As a positive control cells were also stimulated with IL-23 (20ng/mL). As observed previously, IL-23 potently induced innate IL-17A production. However, IL-6 also induced significant production of IL-6 protein ($P<0.05$, Figure 30A).

Since ILCs are the major source of IL-17A in TRUC mice, the role of IL-6 in triggering IL-17A production by this cells population was investigated by flow cytometry. Unfractionated mLN cells were stimulated with IL-6 (50ng/mL) for 4 hours or with medium alone. As a positive control, cells were also stimulated with IL-23 (20ng/mL). Cells were stimulated for a total of 4 hours (the last 2 of which monensin was added). Following stimulation, cells were harvested and ILCs were identified by gating on live, $CD90^+$ $NKp46^-$ cells (Figure 30B). Crucially, in comparison with cells cultured with medium alone, IL-6 induced marked IL-17A production in live, $NKp46^-$ $CD90^+$ ILCs (Figure 31C). As anticipated IL-23, the positive control, also induced IL-17A production in ILCs (Figure 30C). Taken together these data indicate that IL-6, which is increased in TRUC disease, stimulates innate IL-17A production. Furthermore, ILCs which are the major source of IL-17A in TRUC mice express the IL-6R and can be stimulated to produce IL-17A by IL-6.

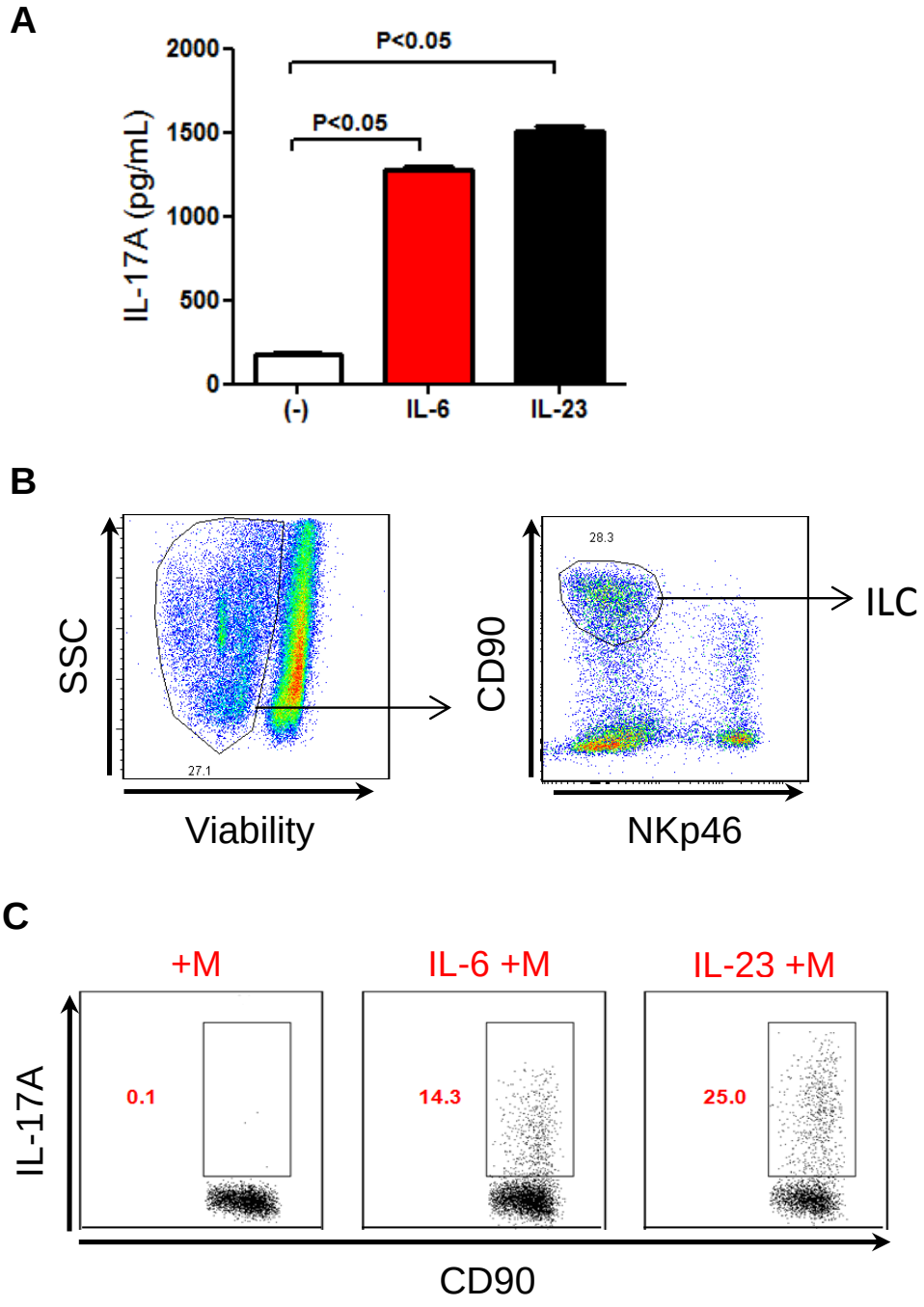

Figure 30. IL-6 induced innate IL-17A expression in TRUC mice. A 1 x 10^5 unfractionated mLN cells from TRUC mice were stimulated with IL-23 (n=3, 20ng/mL) or IL-6 (n=3, 50ng/mL) or incubated with medium alone (n=5), denoted as (-) for 24 hours. Bars represent mean IL-17A concentration in culture supernatants. Error bars denote SEM. B Gating strategy for identifying ILCs in the mLN of TRUC mice. C Intracellular IL-17A production in ILCs following stimulation with IL-23 (20ng/mL) or IL-6 (50ng/mL) or incubated with monensin alone (+M). Cells were stimulated for 4 hours. Monensin was added to stimulated cells for the final 2 hours for each condition, including unstimulated cells. Data are representative of 3 separate experiments.

4.9 Inhibition of STAT3 phosphorylation attenuated innate IL-17A production

In T-cells, ligation of the IL-6 receptor triggers STAT3 phosphorylation (Stahl *et al*., 1994). However, it is unknown whether STAT3 signalling is important in ILC biology. The possibility that IL-6 induced STAT3 phosphorylation might be relevant in TRUC IBD was explored. STAT3 phosphorylation can be specifically inhibited both *in vitro* and *in vivo* by the soluble small molecule inhibitor S3I-201 (Zhang *et al*., 2011). Therefore, a series of experiments was conducted with S3I-201 to determine whether innate IL-17A production in TRUC mice was dependent on STAT3 activation.

Colon explants from TRUC mice were set up in culture in the presence or absence of S3I-201. In contrast to explants incubated with DMSO alone, which produced high levels of IL-17A there was a dose-dependent reduction in IL-17A concentration in culture supernatants when explants were cultured in the presence of S3I-201 (Figure 31A). At a S3I-201 concentration of 20µg/mL there was approximately 50% inhibition of innate IL-17 production ($P<0.05$). At a S3I-201 concentration of 100µg/mL there was approximately 65% inhibition of innate IL-17 production ($P<0.01$).

The role of STAT3 activation in IL-6 induced IL-17A production was also examined. As previously observed IL-6 induced IL-17A production by unfractionated splenocytes ($2x10^6$ cells/mL) from TRUC mice (Figure 31B). However, IL-6 induced IL-17A production was significantly attenuated in the presence of S3I-201 ($P<0.005$, Figure 31B). Taken together these data indicate that innate IL-17A production is at least partly dependent on STAT3 phosphorylation and that inhibition of STAT3 activation reduces IL-6 induced IL-17A production.

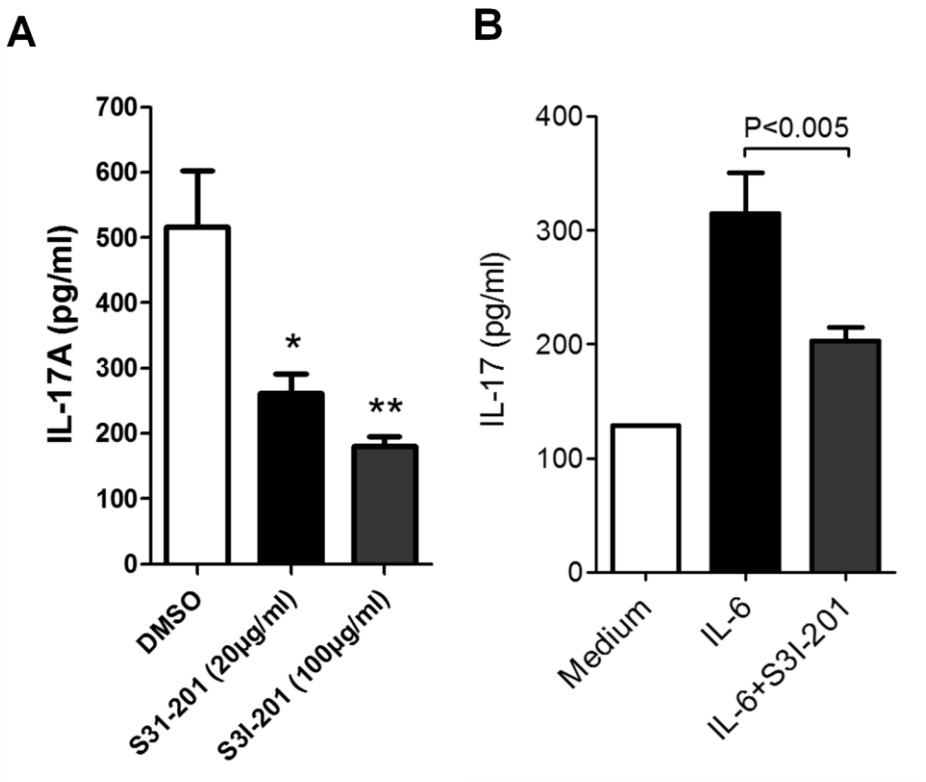

Figure 31. Innate IL-17A production was attenuated by inhibition of STAT3 phosphorylation. A IL-17A concentration in culture supernatants following culture of colon explants from TRUC mice (n=8) Explants were incubated in the presence of medium with DMSO (0.1%) or with different concentrations of an inhibitor of STAT3 phosphorylation (S3I-201) at a final concentration of DMSO 0.1%. *P<0.05, **P<0.01 B Unfractionated splenocytes ($2x10^6$/mL) from TRUC mice (n=8) were stimulated with IL-6 in the presence or absence of S3I-201. Bars denote mean cytokine concentration and error bars denote SEM.

4.10 IL-6 blockade attenuated TRUC disease

IL-6 has been shown to be functionally important in adaptive immune mediated models of IBD, including IBD manifesting in *Il10*$^{-/-}$ (Atreya *et al*., 2000) or SAMP/Yit mice (Mitsuyama *et al*., 2006). Blockade of IL-6 signalling reduces the severity of colitis induced by adoptively transferred naive CD4^{+} T-cells (Kitamura *et al*., 2004) or TNBS administration (Atreya *et al*., 2000). Similarly, IL-6 null mice have less severe colitis and fewer inflammatory cells infiltrating colonic lamina propria following DSS administration (Naito *et al*., 2004). However, in these experiments with adaptive immune mediated models of IBD, the functional importance of IL-6 was typically attributed to its role in driving T-cell responses. The functional importance of IL-6 in innate immune mediated models of colitis is unknown. To determine whether excess IL-6 observed in TRUC mice was functionally important in TRUC disease experiments were conducted in which IL-6 was neutralized *in vivo* with a blocking monoclonal antibody.

Anti-mouse IL-6 (clone: MP5-20F3, Bio X Cell) or control isotype rat IgG1 (clone: HRPN, Bio X Cell) antibodies were administered (750µg) via intraperitoneal injection to age matched, 12 week old TRUC mice on days 0, 4, 9, 14, 18, 23 and 27. This clone has previously been shown to neutralize the biological activity of IL-6 *in vivo* (Starnes *et al*., 1990). Anti-IL-6 was administered to 8 mice and control mAb to 9 mice. Mice were culled on day 29 and organs harvested. One animal (in the control antibody treated group) was withdrawn from the study and humanely culled due to progressive weight loss and severe rectal prolapse.

In comparison with control antibody treatment, administration of anti-IL-6 mAb resulted in significant loss of IL-6 bioavailability in the serum of TRUC mice ($P<0.0002$, Figure 32A). Similarly, IL-6 production was significantly reduced in explant organ culture of anti-IL-6 treated mice ($P<0.015$, Figure 32B).

Crucially, *in vivo* neutralization of IL-6 significantly attenuated disease in TRUC mice. Anti-IL-6 treated mice had significantly reduced colitis histology scores in the distal colon ($P<0.037$, Figure 33A), the site of maximal disease. However, there was no significant difference between histology scores in the proximal colon of anti-IL-6 and control mAb treated mice (Figure 33B). Anti-IL-6 treatment also resulted in a

significant reduction in splenomegaly in TRUC mice ($P<0.007$, Figure 33C). There was a tendency for colonic weight to be reduced in anti-IL-6 treated TRUC mice in comparison with control antibody treated animals, however, this was not statistically significant (Figure 33D). There was no significant change in weight gain/loss between anti-IL-6 or control mAb treated mice (Figure 33E).

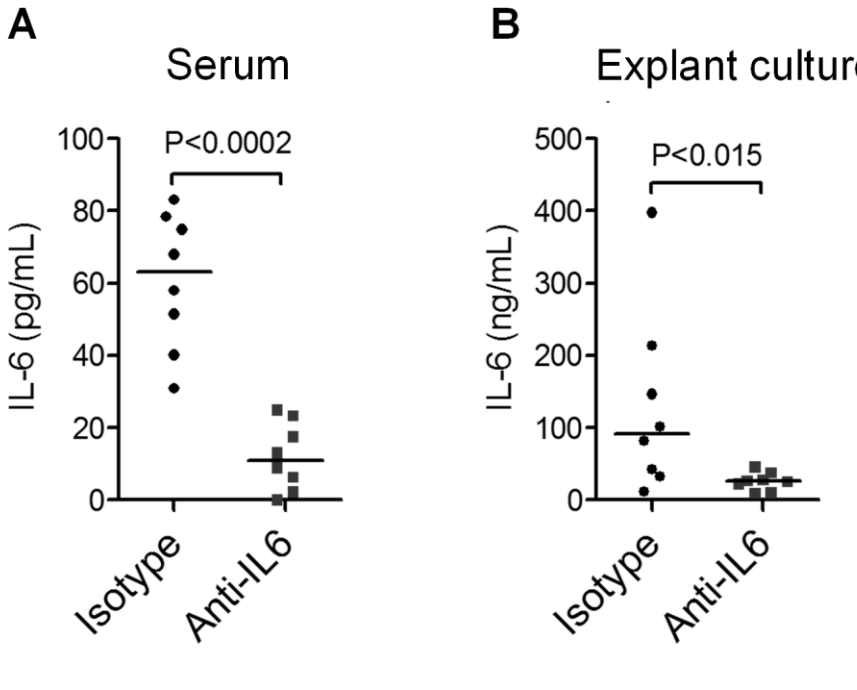

Figure 32. Anti-IL-6 reduced the bioavailability of IL-6 in TRUC mice. A Concentration of IL-6 in the serum of TRUC mice following treatment with an anti-IL-6 mAb (n=8) or control (isotype) antibody (n=8). B Concentration of IL-6 in the explant organ culture of TRUC mice following treatment with an anti-IL-6 mAb (n=8) or control (isotype) antibody (n=8). IL-6 was measured by ELISA. In both graphs lines show median IL-6 concentration.

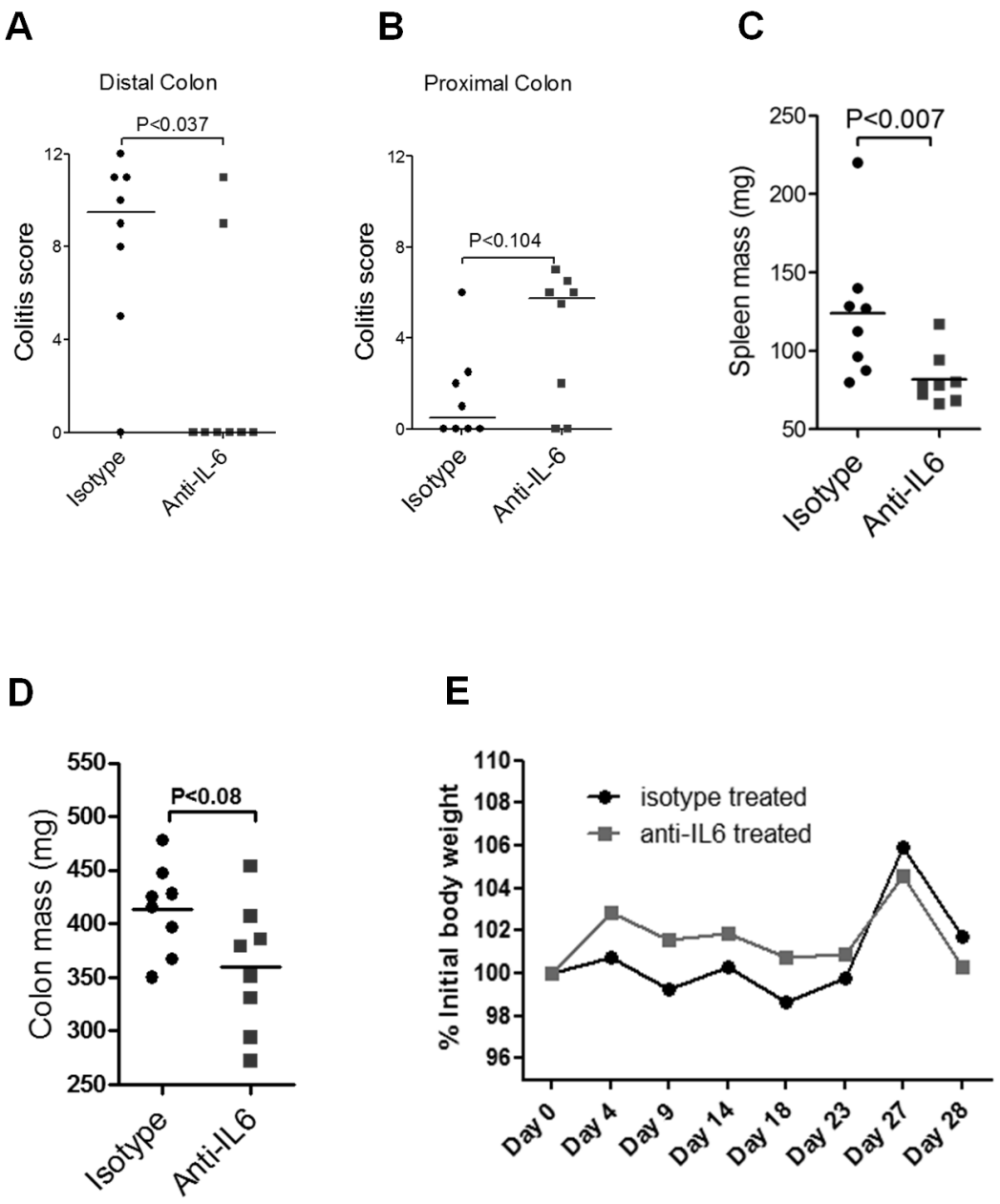

Figure 33. Anti-IL-6 attenuated TRUC disease. A Distal and B proximal colitis histology scores, C spleen mass, D colon mass and E changes in body mass in TRUC mice following treatment with an anti-IL-6 mAb (n=8) or control (isotype) antibody (n=8). In Figure E dots (or squares) represent mean % change in body weight in the treatment groups specified. In Figures A-D each dot represents an individual mouse. Lines depicts median of the variable defined by the y-axis. Note: this was one of last experiments performed during the laboratory phase of this thesis. The acquisition of both distal and proximal colon segments for histological assessment was used as an experimental readout for the first time. Accordingly, this experimental readout was not available in other *in vivo* blockade/depletion studies.

4.11 IL-6 blockade reduced innate IL-17A production in TRUC mice

Since IL-6 was observed to stimulate IL-17A production by ILCs from TRUC mice the hypothesis that IL-6 blockade would reduce innate IL-17A production in TRUC mice was tested. In these experiments, spontaneous production of IL-17A was measured in cells isolated from different organs of TRUC mice following *in vivo* treatment with anti-IL-6 or control isotype antibody.

IL-17A concentration was measured in the serum of TRUC mice treated with anti-IL-6 or control antibody but were undetectable in either group (data not shown). Spontaneous IL-17A production was also measured in culture supernatants of splenocytes harvested from TRUC mice treated with anti-IL-6 or control antibody. Splenocytes were cultured at 2 $x10^6$ cells/mL for 24 hours. Anti-IL-6 treated mice had a significant reduction in spontaneous IL-17 production by splenocytes ($P<0.01$, Figure 34A). Spontaneous production of IL-17A in colon explant culture was also investigated in anti-IL-6 and control antibody treated TRUC mice. The median concentration of IL-17A in explant culture supernatants from anti-IL-6 treated mice was significantly lower than in control antibody treated mice, ($P<0.022$, Figure 34B). Similarly, there was a tendency for reduced spontaneous production of IL-17A by unfractionated mLN cells in anti-IL-6 treated mice in comparison with control antibody treated TRUC mice, although this also did not reach statistical significance ($P<0.083$, Figure 34C). These data support the likelihood that IL-6 is a physiologically important stimulator of innate IL-17A production.

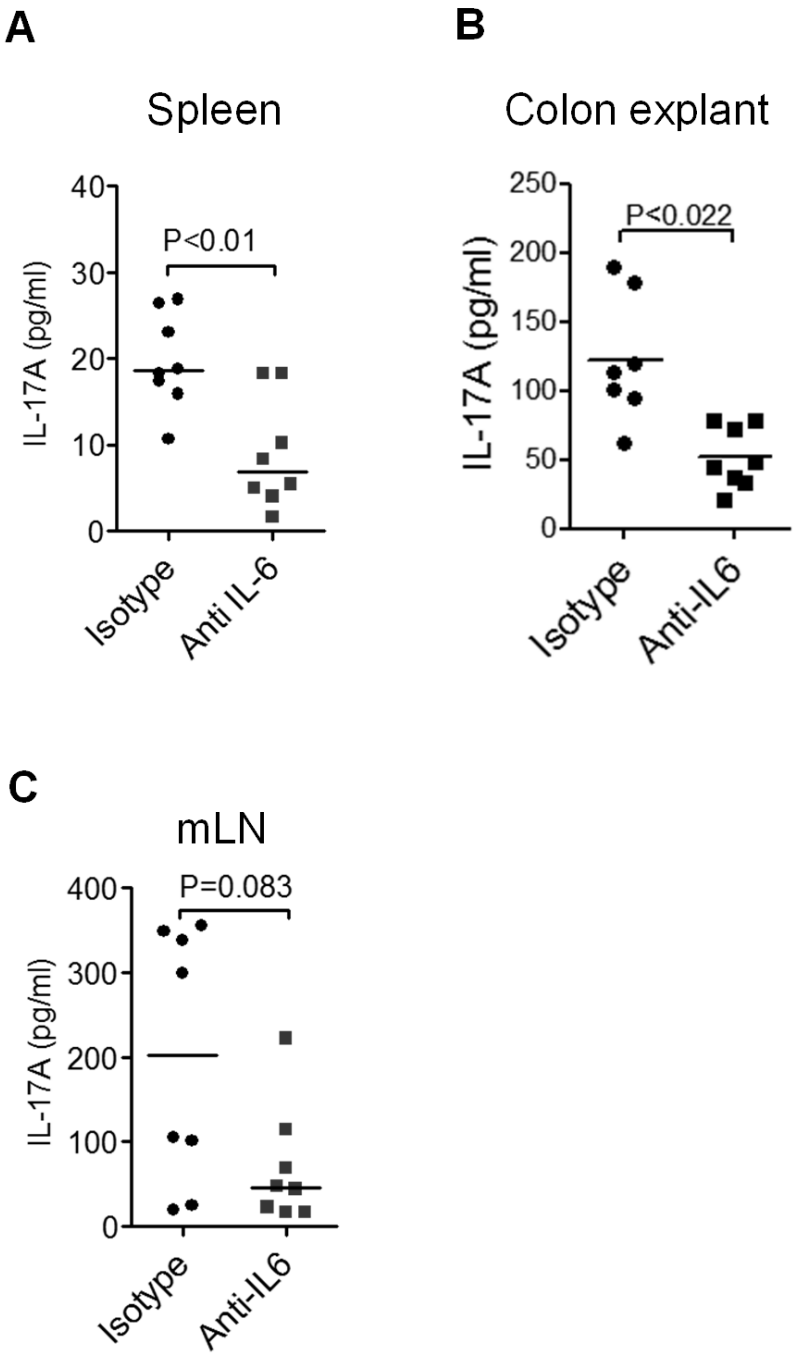

Figure 34. Anti-IL-6 treatment reduced spontaneous IL-17A production in TRUC mice. IL-17A production (measured by ELISA) by A cultured unfractionated splenocytes ($2x10^6$ cells/mL), B Colon explant culture and C unfractionated mLN cells ($1x10^5$ cells/mL) of TRUC mice following treatment with an anti-IL-6 mAb or control (isotype) antibody. This data are from the experiment described in 4.11. Each dot represents an individual mouse. Line depicts median of variable defined by the y-axis.

4.12 IL-6 blockade did not reduce CD69 expression by ILCs from TRUC mice

In other immune cell populations, expression of the cell surface marker CD69 is associated with cellular activation. Therefore, the hypothesis that IL-6 blockade would reduce ILC activation, as measured by CD69 expression, was tested. Although CD69 was highly expressed by intestinal ILCs in TRUC mice (Figure 35A), consistent with an increased activation status of these cells, there was no statistically significant difference between the MFI of CD69 expression in anti-IL-6 or control antibody treated TRUC mice (Figure 35B).

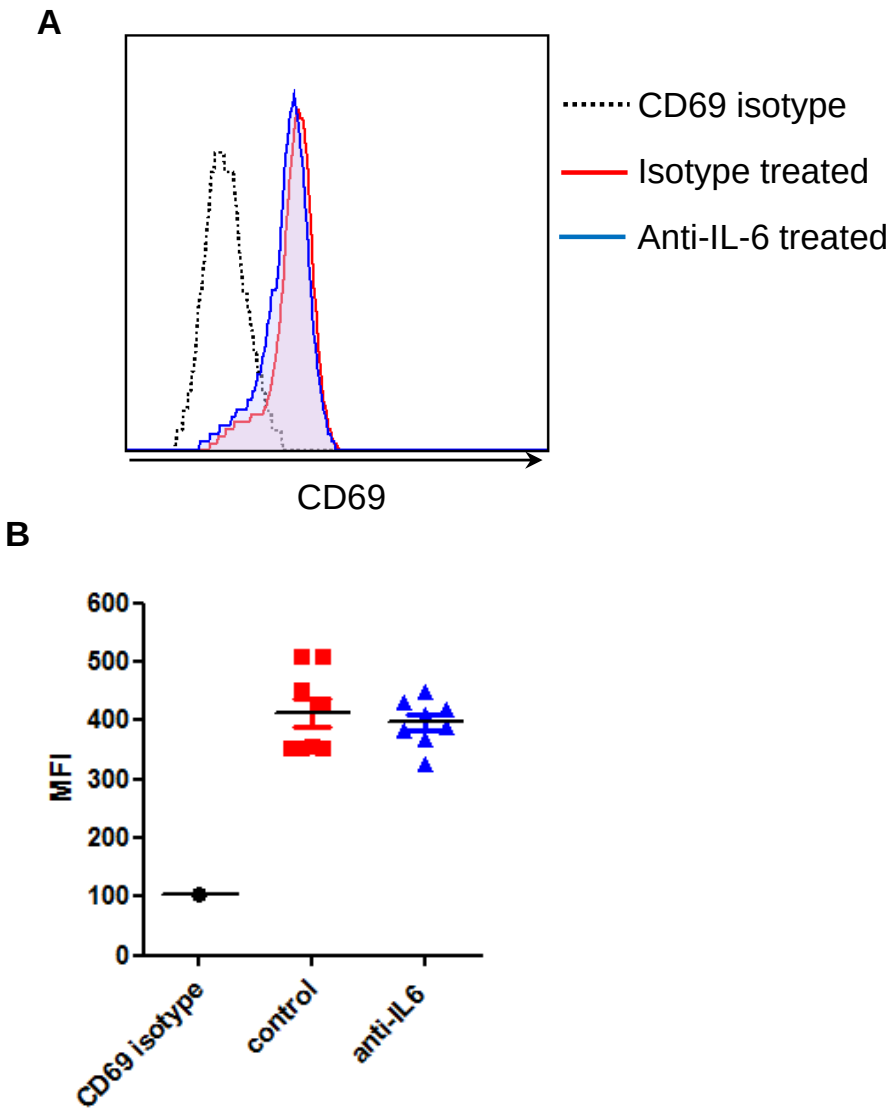

Figure 35. Anti-IL-6 treatment did not reduce CD69 expression on CD90^{+} NKp46^{-} IL-7R^{+} ILCs. A Representative flow cytometry histogram showing CD69 expression (or control isotype staining) in ILCs (gated on NKp46^{-} CD90^{+}) from the cLP of TRUC mice following *in vivo* treatment with anti-IL-6 or control mAb. B Median Fluorescence Index (MFI) of flow histograms comparing CD69 expression in ILCs. Lines depict mean and error bars denote SEM. These data are derived from the experiment described in 4.11.

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4.13 TL1A mRNA expression was increased in the distal colon of TRUC mice

The possibility that TL1A might also impact innate IL-17A responses was considered. The expression of mRNA encoding *Tl1a* in the colon of TRUC and control *Rag2*$^{-/-}$ mice was investigated by qPCR. Colon segments were harvested into Trizol reagent, pending RNA extraction, cDNA synthesis and subsequent qPCR with *Tl1a*–specific primers. mRNA encoding *Tl1a* was significantly increased in the colon of TRUC mice in comparison with *Rag2*$^{-/-}$ mice ($P<0.033$, Figure 36A). Notably, *Tl1a* mRNA expression was significantly increased in the distal colon of TRUC mice in comparison with the proximal colon ($P<0.001$, Figure 36B), which correlates with the most significant site of inflammation histologically in TRUC mice.

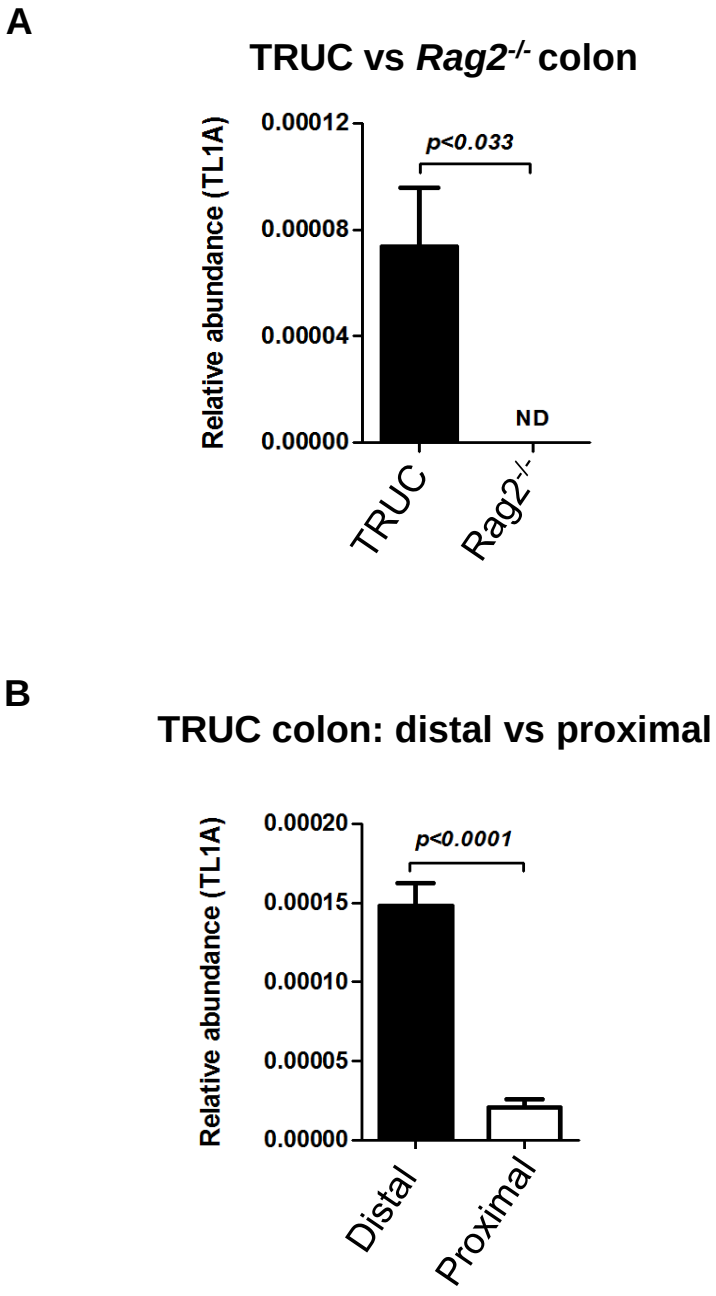


Figure 36. mRNA encoding *Tl1a* was increased in the distal colon of TRUC mice. A Relative abundance of mRNA encoding *Tl1a* in whole colon homogenates from TRUC (n=5) and *Rag2*$^{-/-}$ (n=3) mice measured by qPCR. ND= not detected. B Relative abundance of mRNA encoding *Tl1a* in the distal (0.5cm segment within 0.5cm of the anal verge) and proximal colon (0.5cm segment within 1cm of the caecum) of TRUC mice (n=10) measured by qPCR. ND= not detected. In both graphs bars represent mean relative abundance of cytokine transcript and error bars denote SEM. These data are from a single experiment and no additional experiments were performed.

4.14 ILCs and macrophages from TRUC mice express the TL1A receptor, DR3

To determine which innate immune cells would be capable of responding to TL1A stimulation in the gut, the expression of the TL1A receptor DR3 was investigated in TRUC mice. The expression of DR3 was determined in DCs ($CD11c^{high}$ $classII^{+}$ cells), macrophages ($F4/80^{+}$ cells), NK cells ($NKp46^{+}$) and ILCs ($NKp46^{-}$ $CD90^{+}$) from the mLN of TRUC mice by flow cytometry. Although there was little or no detectable DR3 staining in DCs or NK cells, there was positive staining of ILCs and macrophages (Figure 37).

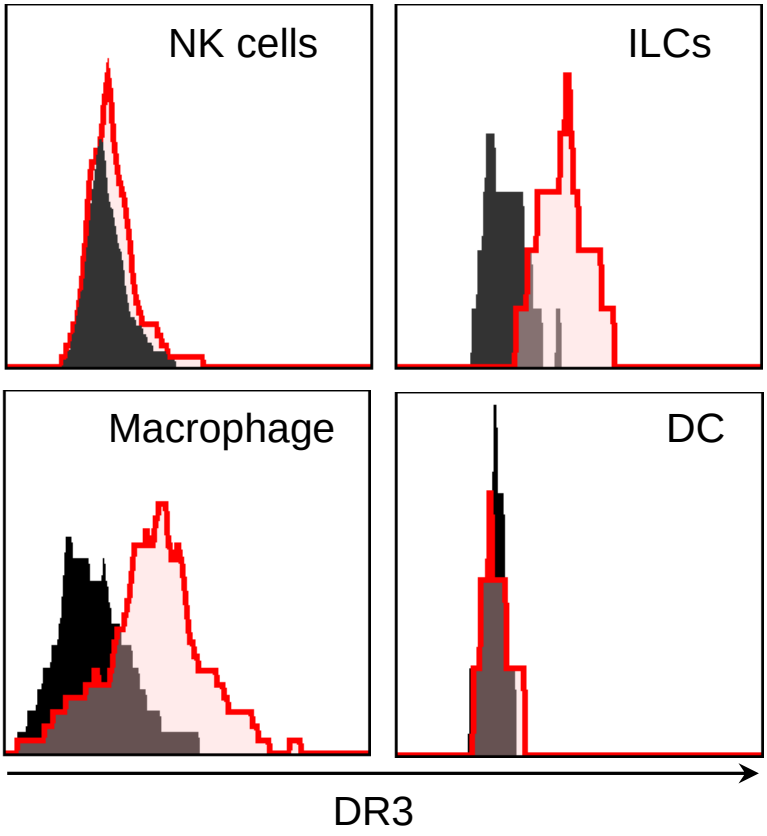


Figure 37. ILCs and macrophages from in the mLN of TRUC mice expressed the TL1A receptor DR3. Flow cytometry histograms showing DR3 staining (red histogram) in comparison with isotype control antibody (black histogram), in NK cells (gated on NKp46^{+} cells), ILCs (NKp46^{-} CD90^{+}), macrophages (F4/80^{+}) and DCs (CD11c^{high} class II^{+}) in the mLN of TRUC mice. These data are representative of 4 biological replicates in a single experiment.

4.15 TL1A augmented IL-23 induced IL-17A production by ILCs

Since mLN ILCs expressed the TL1A receptor, the possibility that these cells may also respond to TL1A stimulation was investigated. Unfractionated mLN cells ($1x10^5$ cells/mL) were cultured in the presence/absence of recombinant murine TL1A and/or IL-23. TL1A induced a significant and dose-dependent increase in the concentration of IL-17A in these cultures (Figure 38A). Most notably, TL1A in combination with IL-23, was the most potent stimulus for innate IL-17A production identified (Figure 39A).

The role of TL1A in triggering IL-17A production by ILCs in TRUC mice was also examined by flow cytometry. Unfractionated mLN cells were stimulated with recombinant TLA1 and/or IL-23. Cells were harvested, stained with surface antibodies (CD90 and NKp46) and then fixed in PFA before being permeabilised and stained with an anti-IL-17A mAb. Unstimulated cells had little or no intracellular IL-17A staining (Figure 38B). However, there was only minor IL-17A production induced by recombinant TL1A by itself (Figure 38B). In contrast, IL-23 induced appreciable intracellular IL-17A production, and most notably the combination of TL1A and IL-23 was highly effective at inducing IL-17A (Figure 38B).

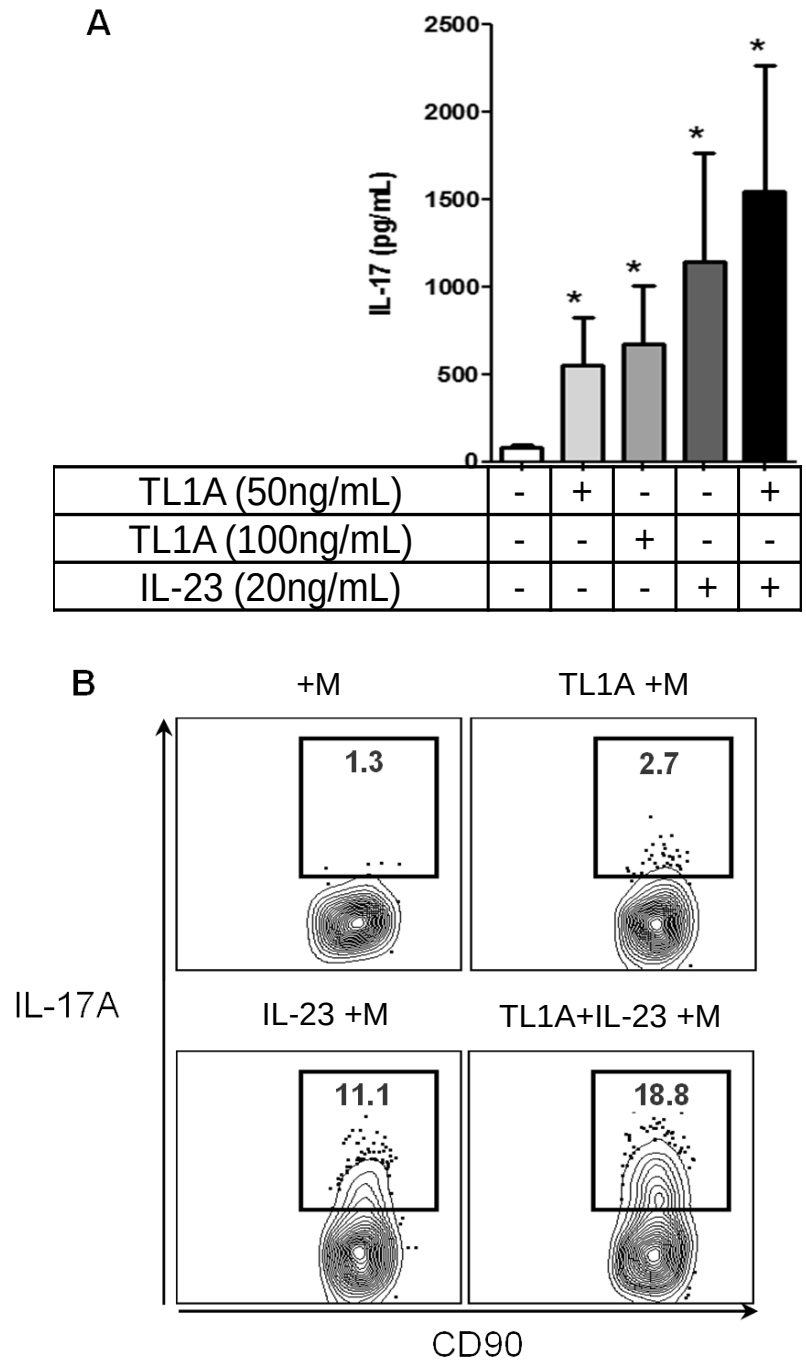


Figure 38. TL1A augmented IL-23 induced IL-17A production by ILCs from TRUC mice. A IL-17A concentration in culture supernatants of unfractionated mLN cells from TRUC mice (n=4) in the presence or absence of combinations of IL-23 and TL1A. Cells were cultured for 24 hours. IL-17A concentration in culture supernatants was measured by ELISA. Bars denote mean IL-17A concentration and error bars denote SEM. *P<0.05 v unstimulated cells. These data are from a single experiment, which was not repeated. B Flow cytometry plots showing intracellular IL-17A expression in ILCs (gated on $NKp46^{-}$ $CD90^{+}$ cells) in mLN of TRUC mice following stimulation with IL-23(20ng/mL) and/or TL1A (50ng/mL). Cells were stimulated for 4 hours. Monensin (+M) was added for the final 2 hours of the stimulation. Unstimulated cells were also cultured in the presence of monensin for the final 2 hours of the experiment. These data are representative 5 biological replicates.

4.16 TL1A blockade failed to attenuate TRUC colitis

Since TL1A mRNA was increased in the colon of TRUC mice, and TL1A was observed to stimulate ILCs, the hypothesis that TL1A was functionally important in TRUC disease was tested. Neutralising anti-TL1A mAbs were kindly provided by Professor Al-Shamkani (Southampton University).

Five hundred µg of anti-TL1A (n=7) or rat IgG2a control antibody (n=9) was administered *i.p.* to 12-14 week old TRUC mice. Antibodies were administered on days 0, 4, 8 and 14. Mice were culled and organs harvested on day 16. At the end of the experiment there was no significant difference in colitis score in the distal colon between mice treated with anti-TL1A or control antibody (Figure 39A). Similarly, there was no significant difference in colon weight of TRUC mice treated with anti-TL1A or control isotype (Figure 39B). Since colitis in TRUC mice tends to be most severe in the distal colon, the weight of the distal 3cm of colon was also measured. However, there was also no significant difference in the mass of the distal colon between anti-TL1A or control isotype treated TRUC mice (Figure 39C). Likewise there was no significant change in weight loss (as a % of initial body mass) between anti-TL1A or control isotype treated TRUC mice (Figure 39D). There was also no significant difference in spleen weight between anti-TL1A or control isotype treated TRUC mice (Figure 39E).

The proportion of different immune cell populations in the colon of TRUC mice following treatment with an anti-TL1A mAb or control isotype was determined by flow cytometry. Colonic LPLs were stained with CD45, viability stain, F4/80 (macrophages), Gr-1 (granulocytes) and CD90 (ILCs). Although there was a tendency for a reduced proportion of granulocytes (median = 4.8%, IQR 1.5-10.0% vs. 10.4%, IQR 3.95%-14.5%, $P<0.094$) and ILCs (median 10.4%, IQR 9.6-14.3% vs. 16.9%, IQR 12.35%-20.93%, $P<0.073$) in the colon of anti-TL1A treated TRUC mice as compared to control antibody treated mice, this did not achieve statistical significance. Taken together these data fail to support the hypothesis that TL1A is functionally important in TRUC disease.

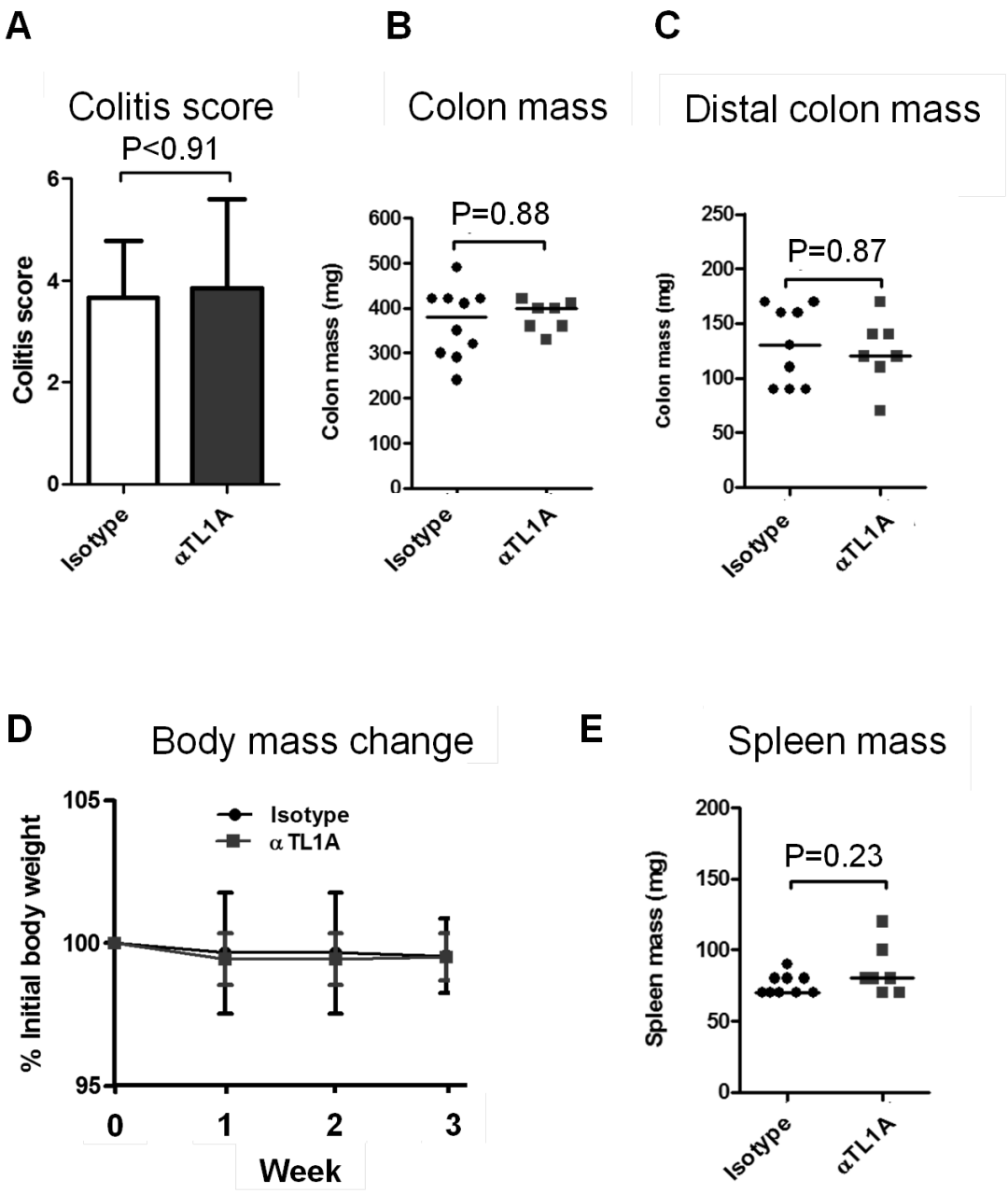


Figure 39. TL1A blockade failed to attenuate colitis in TRUC mice. A Colitis histology scores, B colon mass, C distal 3cm colon mass, D weight loss (as a % of initial body mass) and E spleen mass of 12-14 week old TRUC mice following *in vivo* treatment with anti-TL1A or control isotype antibody. This experiment was conducted on one occasion and was not repeated. In Figures B, C, E each dot represents an individual mouse. Lines depicts median of the variable defined in the y-axis. In Figure A bars represent median colitis score and error bars denote SEM. In Figure D dots denote median body weight (as a % of initial body weight) in control mAb (isotype) or anti-TL1A treated mice. Error bars indicate SEM.

4.17 Discussion

In this chapter some of the proximal cytokine signals responsible for driving ILC activation and chronic colitis have been established. It is shown that IL-23, IL-6 and TL1A triggered IL-17A production by ILCs from TRUC mice.

There is growing evidence implicating IL-23 as an important cytokine in IBD. It has previously been shown that IL-23 is important in driving innate immune mediated colitis caused by *Helicobacter hepaticus* infection in *Rag1*$^{-/-}$ mice (Buonocore *et al*., 2010), therefore the data presented in this thesis support this notion, since IL-23 was also capable of triggering IL-17A production by ILCs in TRUC disease. In this study specific antagonism of IL-23 (rather than IL-12) was therapeutic, which further supports other observations indicating that IL-23 seems to play an important role in colonic rather than systemic inflammation. Indeed, the Powrie group performed an elegant series of experiments to differentiate the relative contribution of IL-12 and IL-23 in gut and systemic inflammation caused by administration of agonistic anti-CD40 mAbs (Uhlig *et al*., 2006). In these experiments *Rag1*$^{-/-}$ mice were crossed with *Il12p40*$^{-/-}$ mice (lacking both IL-12 and IL-23, since the IL-12p40 subunit is shared between these cytokines), *Il12p35*$^{-/-}$ mice (lacking IL-12 but not IL-23, since the IL-12p35 subunit is only a component of IL-12) and *Il23p19*$^{-/-}$ mice (this component is only present in IL-23). Strikingly, *Rag1*$^{-/-}$ *Il23p19*$^{-/-}$ double knockout mice were resistant to colitis but retained sensitivity to systemic inflammation, whereas *Rag1*$^{-/-}$ *Il12p35*$^{-/-}$ double knockout mice were resistant to systemic disease, but remained sensitive to colitis induction. Consistent with these observations *Rag1*$^{-/-}$ mice lacking the IL-12p40 subunit common to both IL-12 and IL-23 were resistant to both colonic and systemic disease. The effectiveness of IL-23 blockade in preventing colonic disease in TRUC mice is consistent with these reports and persuasively supports the employment of strategies targeting IL-23 in IBD patients. To date only anti-IL-12p40 therapies have been evaluated in CD (Mannon *et al*., 2004). The potential advantage of blocking IL-23 alone (rather than dual blockade of IL-23 and IL-12 which occurs with anti-IL-12p40 therapy) is that only colonic inflammation is likely to be targeted and systemic immunity, which might be required for resistance to systemic infections, is left unhindered. Biological therapies targeting IL-23p19

have yet to be reported in IBD, but are due to be published in the near future in clinical trials of patients with other immune mediated diseases, such as psoriasis (unpublished communications with MERCK).

For the first time it was also shown that IL-17A production by ILCs is also triggered by IL-6. ILCs expressed the IL-6R receptors, thus enabling them to respond to excess IL-6 in TRUC disease. The functional importance of IL-6 in TRUC disease was demonstrated by neutralizing this cytokine *in vivo*. Biological neutralization of IL-6 reduced innate IL-17A production in the colon and spleen of treated TRUC mice and reduced colitis score (in the distal colon). Curiously colitis cores in the proximal colon were not significantly affected by IL-6 neutralization. This raised the intriguing possibility that some cytokines (and immune cells) might be differentially regulated regionally along the length of the colon. Alternatively, these observations may speak to the possible temporal differences in the role of different cytokines in the evolution of colitis in TRUC mice over time. In TRUC disease distal colitis scores are consistently higher (indicating more severe disease) in comparison with proximal colitis scores, which are more variable and develop later in the disease process. Interestingly, in TRUC mice the distal colon is most commonly affected by inflammation-associated colorectal cancer (Garrett *et al*., 2007, Garrett *et al*., 2009), a process in which IL-6 has also previously been implicated in the context of microbiota-dependent colitis (Hu *et al*., 2013). The possibility that particular inflammatory cytokines may play a different role in different anatomical sites of the GI tract is hypothetically attractive in view of the characteristic differences observed in the anatomical distribution the different forms of IBD. In UC most patients tend to have distal or left sided disease (distal to the splenic flexure) at presentation, although many of these will progress to more extensive disease over a 10 year period (Solberg *et al*., 2009). Likewise, the distribution of disease is completely different between patients with CD and UC. Notably, in IBD patients and non-inflammatory controls there is a gradient of gene expression, including inflammatory cytokines across the distribution of the colon (Noble *et al*., 2008), consistent with the possibility that the molecular drivers of disease may be anatomically separable. Further studies will focus on molecular and cellular differences between the proximal and distal colon in early and late TRUC disease to try and tease out the basis for

some of the interesting differences in disease evolution and response to IL-6 neutralization.

It is also shown for the first time that IL-6 likely triggers pSTAT3 activation in ILCs and that inhibition of STAT3 reduces innate IL-17A production, including IL-6 induced IL-17A production in disease relevant tissue in TRUC mice. However, it should also be acknowledged that IL-23 also acts via the STAT3 pathway (at least in T-cells) (Parham *et al*., 2002), therefore, some of the inhibition of spontaneous innate IL-17A seen in explant organ culture experiments could also be due to limitation of IL-23 induced STAT3 phosphorylation. These interesting novel data potentially afford new insights into some of the lessons learned from genetic studies. It is possible that polymorphisms at the *STAT3* locus identified as IBD risk conferring mutations could potentially impact IL-6 mediated innate IL-17A production in IBD (Jostins *et al*., 2012). Indeed, the *IL6ST* gene, which encodes the key IL-6R signal transducing protein is also associated with altered IBD risk (Jostins *et al*., 2012). These data also support the notion that the role of IL-6 in supporting adaptive IL-17A responses is conserved in the primitive innate immune system. Just as monoclonal antibodies targeting IL-12p40 are emerging in to clinical practice for IBD patients (Mannon *et al*., 2004), these data support the rationale for IL-6 neutralization as a tractable therapeutic option in some IBD patients. It is tempting to speculate that in the future it will be possible to stratify IBD patients according to their predominant effector cytokine pathway. Fewer than 50% of patients with IBD achieve robust remission (mucosal healing) with anti-TNFα therapies (Colombel *et al*., 2010), indicating that most patients have TNFα-independent mucosal lesions that are presumably driven by other inflammatory cytokines, such as IL-6 or IL-1β. Accurate ways of stratifying patients according to their prevailing cytokine response, perhaps by explant organ culture or mucosal gene expression profiling may herald a new way of identifying which patients are likely to benefit from which biological therapies targeting a particular cytokine.

Just like IL-6 and IL-23 cytokine pathways there are also polymorphisms at the TL1A locus which confer altered risk of IBD (Jostins *et al*., 2012). In the present study it is shown that ILCs express the TL1A receptor DR3 in TRUC mice and accordingly these cells are potential responders to the increased expression of TL1A

observed in the colon of TRUC mice. Notably, *Rag2*$^{-/-}$ mice with non-inflamed colons have undetectable *Tl1a* mRNA, whereas this cytokine is enriched in the colon of TRUC mice. Similarly, *Tl1a* is also upregulated in the distal colon of TRUC mice in comparison with the proximal colon, correlating with the site of more severe inflammation in TRUC mice. These data mirror the situation in human disease. mRNA encoding TL1A is enriched in inflamed sigmoid colon biopsies in comparison with non-inflamed sigmoid colon biopsies from UC patients (Noble *et al*., 2008).

Although only a weak inducer of IL-17A by itself TL1A synergized with IL-23 to potently induce IL-17A production by ILCs in TRUC mice. It should be noted that experimental blockade of TL1A in TRUC mice was potentially suboptimal in this series of experiments due to limited availability of neutralizing antibody (which was a kind gift, but in short supply). For example, in contrast to blockade of IL-17A, in which treated mice received 7 doses of antibody over the treatment regime, TRUC mice treated with anti-TL1A only received 4 doses. Therefore, it is not possible to be certain that TL1A was effectively blocked *in vivo*, and consequently the *in vivo* functional relevance of TL1A in driving innate IL-17A responses cannot be reliably or conclusively defined by this set of experiments. Further work and additional *in vivo* blockade experiments (or crossing of TRUC mice with *Tl1a*$^{-/-}$ or *Dr3*$^{-/-}$ mice) will be needed to definitely answer this question. Additional experiments might also investigate how TL1A enhances IL-23 mediated IL-17A production by ILCs. Possible mechanisms of TL1A-augmented IL-23-induced IL-17A production by ILCs might include upregulation of the IL-23R on ILCs (thereby increasing IL-23 mediated cell activation) or upregulation of other signalling components downstream of the IL-23 receptor (e.g. STAT3, JAK2, TYK2). Indeed, TL1A-triggered DR3 signalling has been shown to induce other cytokine receptors, such as the IL-2 receptor components CD25 and CD122 in T-cells, which results in increased sensitivity of T-cells to IL-2 (Meylan *et al*., 2011). Therefore, the possibility that TL1A might increase the expression of other cytokine receptors, such as IL-23R (and hence responsiveness to IL-23) is biologically plausible.

In conclusion, the functionally important proximal signals responsible for ILC activation in TRUC disease are IL-23 and IL-6. It is interesting that these cytokines

are implicated in promoting the differentiation of Th17 cells, indicating that these cytokines play an evolutionary conserved role in driving both adaptive and innate IL-17A responses. Therapies aimed at blocking these cytokines *in vivo* have shown promise in clinical trials in IBD patients (Ito *et al*., 2004; Mannon *et al*., 2004). It is possible that some of the clinical efficacy observed with these agents in IBD might correspond to the limitation of ILC activation. Currently, there are no data available regarding TL1A blocking drugs in IBD or indeed other autoimmune diseases. One might argue that irrespective of its function on ILCs, antagonism of the TL1A/DR3 interaction remains an attractive therapeutic target, since TL1A is an important driver of pathogenic T-cell responses (Bamias *et al*., 2003), which also undoubtedly play an important role in IBD. It is arguable that targeting cytokine networks which act on both innate (ILC) and adaptive (T-cell) immune cells might be an especially advantageous strategy to prevent redundancy, where one arm of the immune system emerges as an effector pathway when the other is therapeutically antagonised. With this notion in mind, blockade of IL-6 or IL-23, which can now be acknowledged as cytokines which potently drive both innate and adaptive immune responses, are potentially attractive therapeutic targets in IBD.

CHAPTER 5

Results: Defining the microbial triggers responsible for driving IBD in TRUC mice

The intestinal microbiota is strongly implicated in IBD. No definite causative organisms have been identified in IBD, although important overall changes in the gut microbiota community composition include reduced biodiversity and probable expansion of usually low abundance phyla, such as *Proteobacteria* and even *Actinobacteria* (Frank *et al*., 2007; Lepage *et al*., 2011). In TRUC mice, colitis is dependent on the microbiota, since modulation of the microbiota with antibiotics (Garrett *et al*., 2010) or probiotics (Veiga *et al*., 2010) attenuates disease, and when *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice are reared in germ free conditions they do not get disease (Garrett *et al*., 2010). In view of the central importance of the intestinal microbiota in TRUC disease, and indeed in IBD, the work in this chapter endeavoured to determine whether there were any specific components of the intestinal microbiota of TRUC mice that were responsible for driving disease.

5.1 Newly derived *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice did not develop spontaneous colitis

At the beginning of this programme of research, a new colony of *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice was generated by breeding newly purchased *Rag2*$^{-/-}$ and *Tbx21*$^{-/-}$ mice. The newly derived *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ colony was maintained in specific pathogen free (SPF) isolators, and unexpectedly mice from this new colony failed to develop disease. Histological assessment of the distal colon following H&E staining of mice from this new colony failed to show any evidence of colitis (Figure 40A). The macroscopic appearance of the colon of mice from the new colony was also markedly different from that of TRUC mice, with a lack of colonic thickening, especially apparent in the distal colon (Figure 40B). To distinguish this new colony of non-colitic *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice from their isogenic TRUC counterparts we termed these animals TRnUC (*Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ non-ulcerative colitis) mice. In

keeping with an absence of disease the colon weight of TRnUC mice was significantly less than that of age matched TRUC mice ($P<0.0001$, Figure 40C). In contrast, there was no significant difference in the colon weight of TRnUC mice in comparison with *Rag2*$^{-/-}$ mice (Figure 40C). Similarly, spleen weight of TRUC mice was significantly greater than age matched TRnUC mice (Figure 40D).

This new colony of disease free mice demonstrated that T-bet deficiency in the innate immune compartment (in the absence of adaptive immunity) by itself is insufficient to induce colitis. Furthermore, the availability of isogenic *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ colonies with different phenotypes provided an opportunity to define the environmental trigger responsible for colitis in TRUC mice.

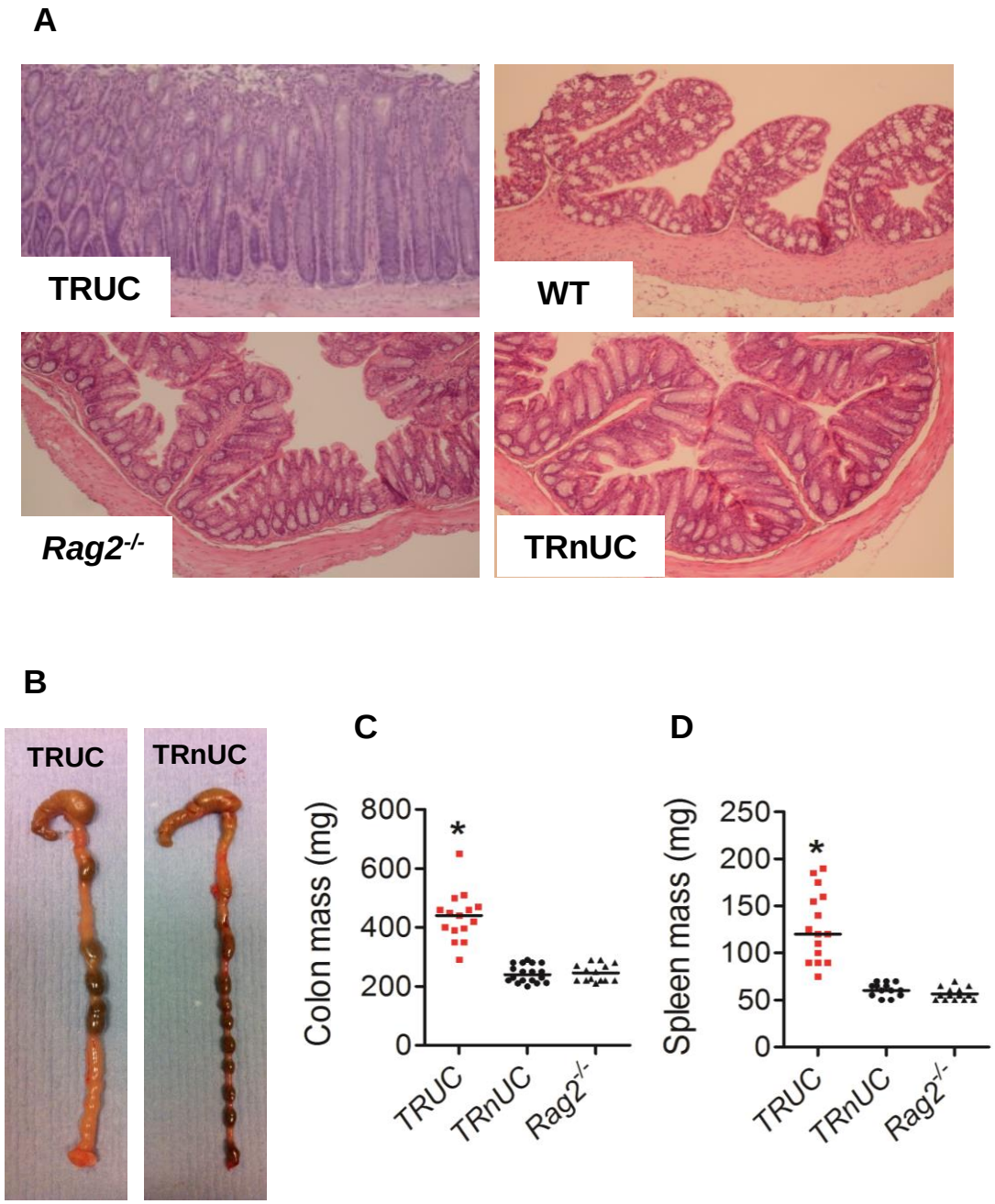


Figure 40. Newly derived *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice failed to develop spontaneous colitis. A Histology of the distal colon of TRUC, TRnUC (newly derived *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ non-ulcerative colitis), *Rag2*$^{-/-}$ and WT mice. Photographs are all taken at the same magnification (approximately x40). B Macroscopic appearance of the colon of TRUC and TRnUC mice. C Colon and D spleen mass of age matched TRUC, TRnUC and *Rag2*$^{-/-}$ mice. *P<0.0001). Each dot represents an individual mouse. Lines depicts median of the variable defined in the y-axis. Adapted from Powell *et al*., 2012.

5.2 16S ribosomal RNA gene sequencing of the intestinal microbiota showed differences between the colonic bacterial community profiles of TRUC and TRnUC mice

Since antibiotics and probiotics are therapeutic in TRUC colitis (Garrett *et al*., 2010; Garrett *et al*., 2007; Veiga *et al*., 2010), and intestinal inflammation is communicable (Garrett *et al*., 2007), the most likely colitogenic stimulus in TRUC disease was an intestinal microbe. Therefore the intestinal microbiota of isogenic TRUC and TRnUC mice was compared by performing 454 pyrosequencing of the V3-V5 regions of bacterial 16S rRNA genes in faecal samples from TRUC and TRnUC colony members.

Fresh stool samples were harvested from TRUC and TRnUC colony members and immediately frozen pending transportation on dry ice to the Sanger Institute in Cambridge, where Dr Alan Walker and Professor Julian Parkhill sequenced and profiled the bacterial 16S ribosomal RNA genes present in these samples. Bacterial DNA was extracted and PCR-amplicons were generated for 454 sequencing using barcoded primers targeting the V3-V5 regions of the 16S rRNA gene. PCR products were then quantified using a Qubit® 2.0 Fluorometer (Invitrogen). After processing, and subsequent manual removal of suspect or chimeric operational taxonomic units (OTUs), 33528 sequences remained, which were split into 256 OTUs overall. The median number of sequences per sample was 2817 (range 1781-3884). OTUs that were significantly differentially abundant between the TRUC and two TRnUC mouse cohorts were identified using the Metastats program (Schloss *et al*., 2009; White *et al*., 2009), allowing differences in the community structures of the different colonies to be identified at the level of both phylum and species.

Stool samples from TRUC mice (n=4) and from 4 mice each from 2 colonies of TRnUC mice were analysed (8 in total). One of the disease free TRnUC colonies was housed at the biological services unit at King's College London (KCL) and the second disease free colony of TRnUC mice was housed at a geographically separate isolator at Charles River laboratories (CRL). This second colony of TRnUC mice was derived from the initial founder TRnUC colony at KCL, but was relocated to CRL to preserve the non-colitis phenotype.

Individual samples shared broad similarities in community structure with other members of the same colony but were distinct from mice in the other colony (Figure 41). As expected, all samples were dominated at the phylum level by *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria* and *Deferribacteres* (Figure 42A). However, the proportional abundance of sequences from the *Proteobacteria* phylum was significantly increased in TRUC mice (Figure 42B), and the ratio of *Firmicutes* to *Bacteroidetes* was significantly higher in TRUC compared to TRnUC mice (Figure 42C).

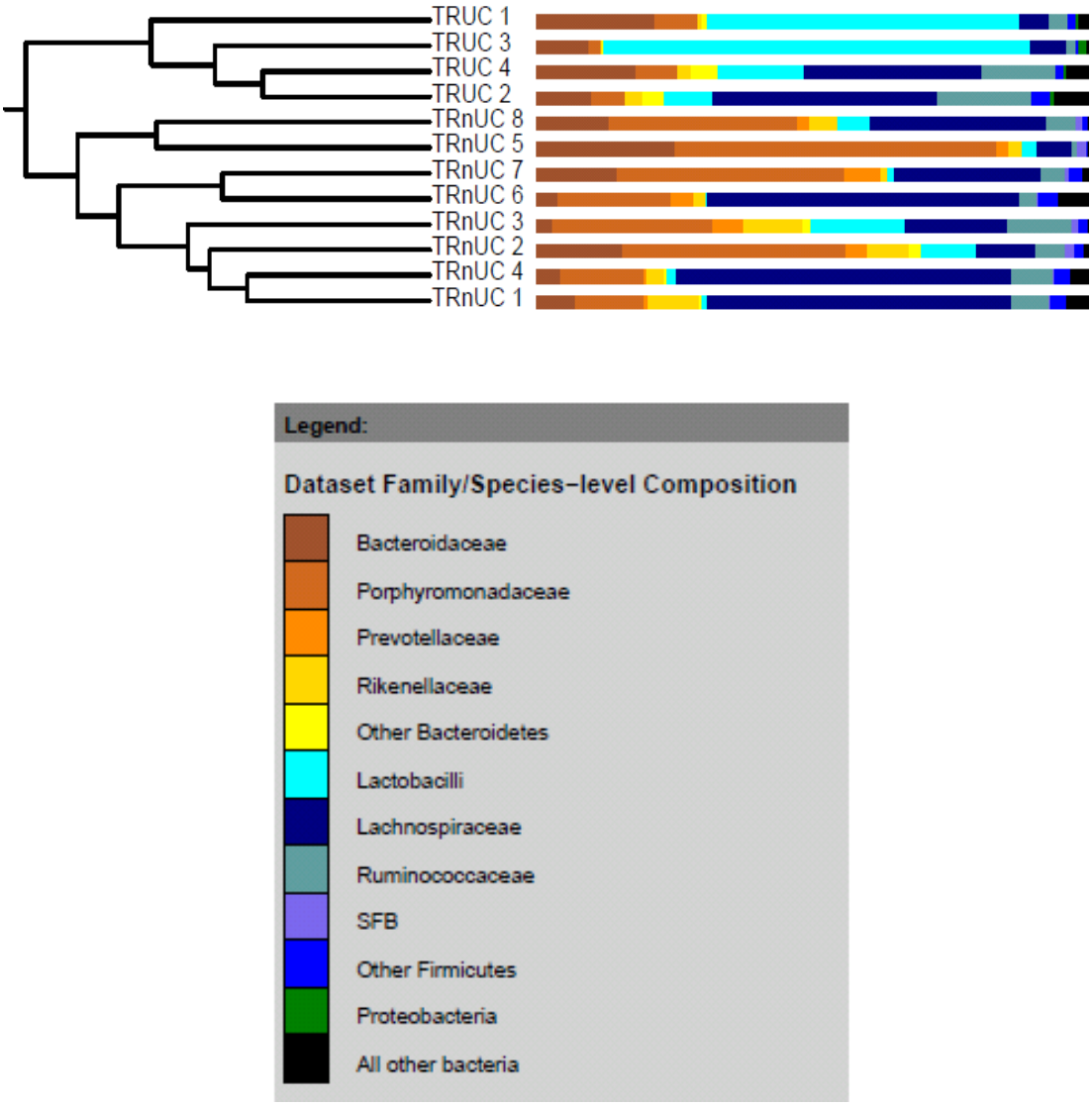


Figure 41. Intestinal microbiota community structure of *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice was different in mouse colonies with and without colitis. Cluster dendogram showing relative family/species level composition of TRUC mice with colitis (TRUC 1-4) and TRnUC mice without colitis from KCL (TRnUC 1-4) and CRL (TRnUC 5-8). These data were generated by Alan Walker and Julian Parkhill. Adapted from Powell *et al*., 2012.

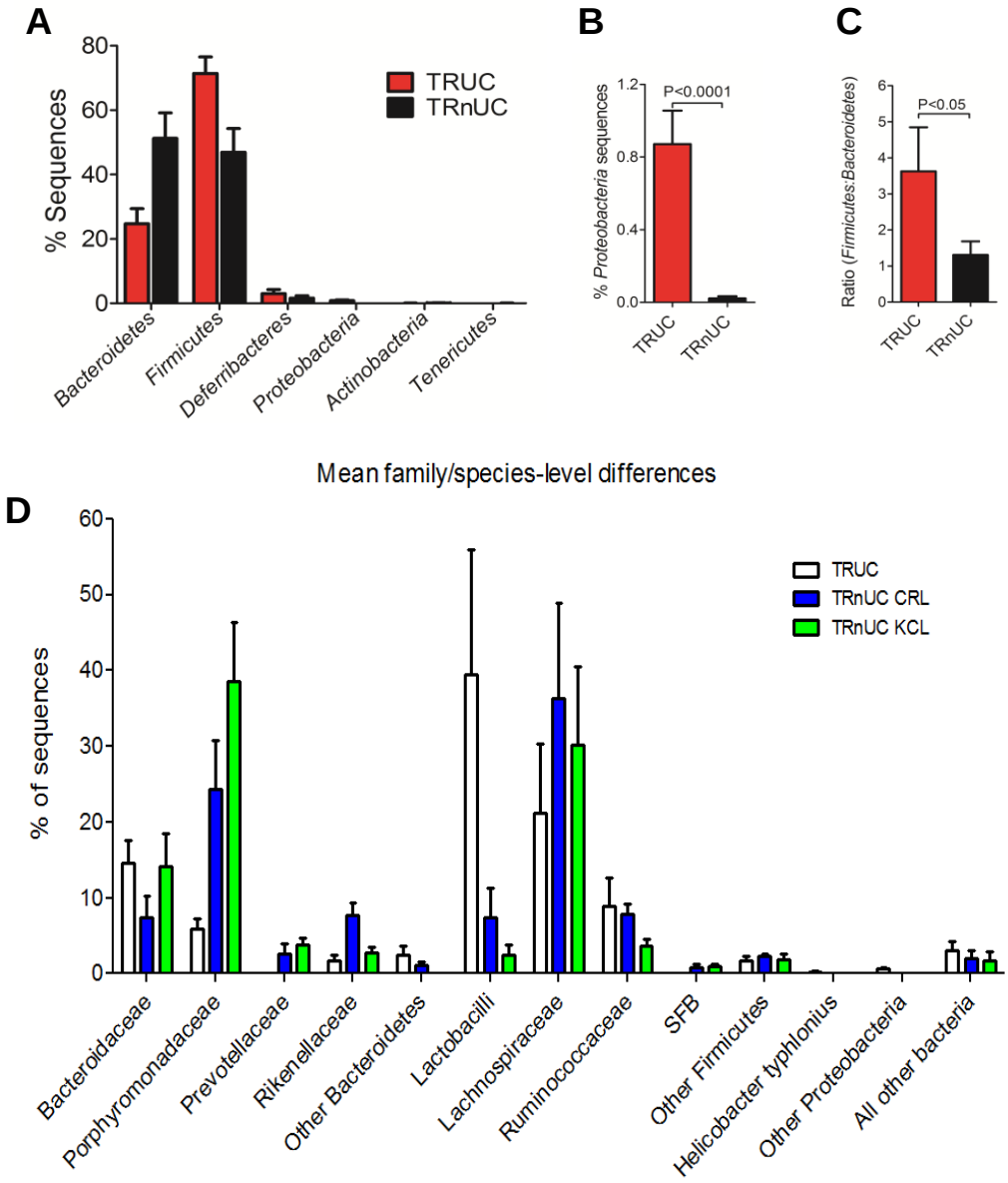


Figure 42. Phylum/family level differences in the intestinal microbiota of TRUC and TRnUC mice. A-C Mean phylum level differences in the intestinal microbiota from TRUC (n=4) and TRnUC (n=8) mice by sequencing bacterial 16S rRNA genes. Error bars indicate SEM**.** Mice were aged 6-8 weeks old. Mice from the 2 separate TRnUC colonies (one at KCL and one at Charles River UK) had been separated for 14 months. In this experiment TRnUC mice from the 2 colonies were combined to generate sufficient statistical power to compare with individual Phyla frequencies from TRUC mice. D Proportion of family/species-level sequences in the different genotypes, including an analysis of the 2 different TRnUC colonies (n=4 in each group). Bars show mean % sequences and error bars show SEM. SFB= Segmented filamentous bacteria. Adapted from Powell *et al.*, 2012.

5.3 Species level differences in TRUC and TRnUC mice

Analysis of the family level differences in the relative frequency (% of sequences) of selective families failed to demonstrate any significant differences between the TRUC colony and the 2 TRnUC colonies (Figure 42D). However, it should be noted that with only 4 mice in each group statistically significant differences might be potentially difficult to detect. However, in an alternative approach the experiment was analysed by trying to determine species-level differences between the colonies to see if any particular species were always present in mice with disease (e.g. TRUC mice), but consistently absent from disease-free TRnUC mice from either colon. In this experiment the faecal microbiota from the 2 colonies TRnUC mice were grouped together as they shared a disease-free phenotype. Analysis of species-level OTUs demonstrated that there were a total of 256 OTUs detected across the intestinal microbiota of all of the TRUC and TRnUC colony members. However, just 12 species-level OTUs were consistently present in all TRUC mice and always absent from TRnUC mice (Figure 43A).

Interestingly, one of the TRUC-specific OTUs matched with 100% identity to the proteobacterial species *Helicobacter typhlonius*. Notably, a number of different *Helicobacter* species have previously been linked to colitis development in research mice (Fox *et al*., 2011). To confirm that *H. typhlonius* was present in TRUC colony members and absent from TRnUC mice, *H. typhlonius*-specific PCR (Feng *et al*., 2005) was performed on bacterial DNA isolated from faeces. PCR using *H. typhlonius*-specific primers confirmed the presence of this bacterium in all TRUC colony members tested, but in none of the TRnUC colony (Figure 43B).

A

NCBI BLAST ID	Phylum	Family	Genus	p value
Helicobacter typhlonius	P	*Helicobacteraceae*	*Helicobacter*	$<2x10^{-9}$
Parasutterella excrementihominis	P	*Alcaligenaceae*	*Parasutterella*	$<1x10^{-10}$
Parabacteroides distasonis	B	*Porphyromonadaceae*	*Parabacteroides*	$<1x10^{-10}$
Bacteroides splanchnicus	B	*Porphyromonadaceae*	*Odoribacter*	0.012
Alistipes shahii	B	*Rikenellaceae*	*Alistipes*	$<1x10^{-10}$
Rikenella microfusus	B	unclassified	unclassified	$<1x10^{-10}$
Anaerotruncus colihominis	F	*Ruminococcaceae*	*Acetanaerobacterium*	$<5x10^{-10}$
Coprobacillus cateniformis	F	*Erysipelotrichaceae*	*Coprobacillus*	$7x10^{-5}$
Eubacterium plexicaudatum	F	*Lachnospiraceae*	unclassified	$<6x10^{-5}$
Clostridium algidixylanolyticum	F	*Lachnospiraceae*	Unclassified *Lachnospiraceae*	<0.0004
Clostridium algidixylanolyticum	F	*Lachnospiraceae*	Unclassified *Lachnospiraceae*	$<3x10^{-10}$
Clostridium piliforme	F	unclassified	unclassified	$<1x10^{-10}$

B

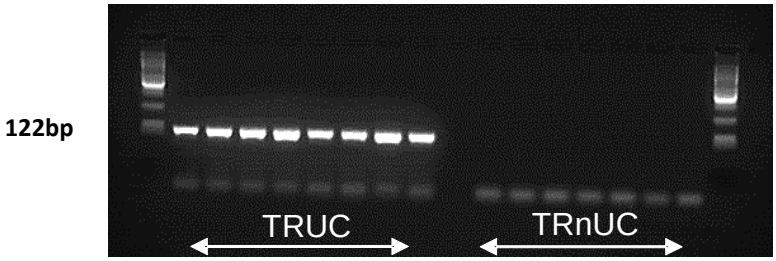


Figure 43. Bacterial species present in the colon of TRUC mice and consistently absent from non-colitic TRnUC mice. A Species level (OTU) bacteria present in TRUC, but absent from TRnUC mice following 434 sequencing of bacterial rRNA genes. TRnUC mice from both colonies were included in this analysis – since both colonies were disease-free and therefore, served as appropriate controls for mice with a colitis phenotype. Phylum Key: P = *Proteobacteria*, B = *Bacteroidetes*, F = *Firmicutes*. Phylum, Family and Genus classifications were generated using the RDP Classifier tool (Cole et al., 2009). B 2% Agarose gel electrophoresis of PCR products following *Helicobacter typhlonius* specific PCR performed on bacterial DNA isolated from fresh faecal samples from TRUC and TRnUC mice (from the Charles River colony). These data are representative of similar experiments with TRnUC mice from the KCL colony which were also negative for H. typhlonius by PCR. The *Helicobacter typhlonius* specific PCR product is 122bp. Adapted from Powell *et al*., 2012.

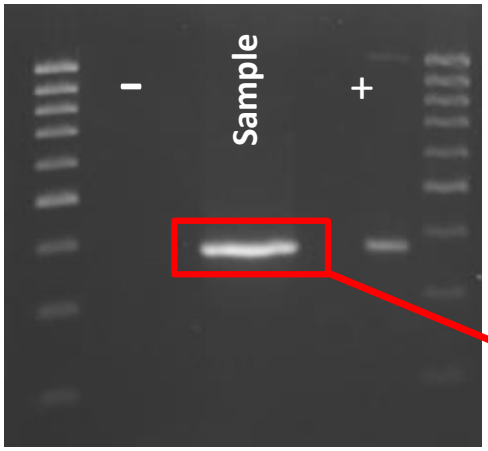
5.4 There were no other recognised pathogens present in the intestinal microbiota of TRUC mice

In an alternative approach, a high sensitivity PCR-based screening test was used to evaluate for the presence of other recognized pathogens in faecal samples acquired from TRUC and TRnUC. This commercially available assay (Prevalent Rodent Infectious Agent, or PRIA panel, Charles River Laboratories, UK) was conducted by an outside vendor (CRL), blinded to the earlier 16S rRNA gene sequencing results. The panel screens for important intestinal pathogens, and utilizes genus-generic primer sets for *Helicobacter*, *Salmonella* and *Campylobacter*, species-specific primers for *Helicobacter hepaticus*, *Helicobacter bilis*, *Citrobacter rodentium*, murine norovirus and rotavirus, and numerous additional well-recognized murine pathogens (Figure 44). No pathogens were identified in faecal samples from TRnUC colony members. In contrast, the *Helicobacter* genus-generic primers confirmed the presence of a *Helicobacter* species in the faecal samples from TRUC mice. However, species-specific PCR for *Helicobacter hepaticus and Helicobacter bilis* were negative, indicating that the Helicobacter species present was neither of these recognised pathogens. To confirm the identity of the *Helicobacter* present in TRUC faeces, a second *Helicobacter* genus generic PCR was performed. The PCR was performed together with a positive control (*Helicobacter hepaticus* DNA). The Helicobacter genus generic primers amplified a 374bp 16S rRNA gene PCR product (Figure 45A), which was subsequently excised from the agarose gel for DNA sequencing (performed by an outside vendor – CRL). The sequence generated is shown in Figure 45B. The sequence was compared with archived sequences in the NCBI GenBank database and with the amplicons generated using the universal bacterial primer sets for 454 sequencing. This analysis confirmed that the *Helicobacter* present in the faeces of TRUC mice shared >99% 16S rRNA gene sequence similarity with *H. typhlonius*.

	TRUC	TRnUC
Helicobacter genus generic	+	-
Salmonella genus generic	-	-
Campylobacter genus generic	-	-
Helicobacter hepaticus	-	-
Helicobacter bilis	-	-
Citrobacter rodentium	-	-
Pasteurella pneumotropica	-	-
Staphylococcus aureus	-	-
Pseudomonas aeruginosa	-	-
Campylobacter genus generic	-	-
Streptobacillus moniliformis	-	-
Clostridium piliforme	-	-
Corynebacterium bovis	-	-
Corynebacterium kutscheri	-	-
Bordetella bronchiseptica	-	-
Mycoplasma pulmonis	-	-
Klebsiella pneumoniae	-	-
Streptococcus pneumoniae	-	-
Streptococcus agalactiae	-	-
Murine norovirus	-	-
Murine rotavirus	-	-
Parvovirus (MPV-1-4, and MVM)	-	-
Adenovirus (MAV-1 and MAV-2)	-	-
Mouse hepatitis virus	-	-

Figure 44. PRIA panel testing of the faeces of TRUC and TRnUC mice. Adapted from Powell *et al.*, 2012.

A



374bp PCR product (for sequencing)

B

5-GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAA
5-GTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTACTCGGAATCACTGGGCGTAAA

GAGTGCGCAGGCGGGGTGTGTAAGTCAGATGTGAAATCCTGTAGCTTAACTACAGAACTG
GAGTGCGCAGGCGGGGTG -GTAAGTCAGATGTGAAATCCTGTAGCTTAACTACAGAACTG

CATTTGAAACTATCACTCTAGAGT-3'
CATTTGAAACTATCACTCTAGAGT-3'

Genus generic Primers 144 (black)
Universal 16S rRNA gene Primers 143 (green)

Figure 45. The DNA sequence of the *Helicobacter* species present in the faeces of TRUC mice matched the sequence of *H. typhlonius*. A 2% Agarose gel electrophoresis of a *Helicobacter* genus-generic PCR performed on a fresh faecal sample from 12 week old TRUC mouse. The band was excised and DNA sequenced. Positive +and negative - controls are illustrated. The sequence of the PCR product (black sequence) was compared with archived DNA sequences in the NCBI GenBank database and with the amplicons generated using the universal bacterial primer sets for 454 sequencing (green sequence), which confirmed that the *Helicobacter* present in the faeces of TRUC mice shared >99% 16S rRNA gene sequence similarity with *H. typhlonius*. Adapted from Powell *et al*., 2012.

5.5 Intestinal colonization with *H. typhlonius* transmitted colitis to TRnUC mice

Since *H. typhlonius* (HT) was present in all diseased mice and absent from healthy mice, the hypothesis that HT might transmit colitis to TRnUC mice was tested. Pure lyophilized cultures of HT (CCUG 48335 T) were acquired from the Culture Collection of the University of Gothenburg (Franklin *et al*., 2001). Bacteria were grown on horse blood agar or Columbia agar and growth occurred under microaerophilic or anaerobic conditions, generating small translucent colonies. One-5 x10^7 organisms were gavaged in to TRnUC mice. As a control, some mice were gavaged with broth alone without the addition of HT. A faecal sample was acquired from test mice at baseline (time 0, prior to gavage) and at 1 week post gavage, 2 weeks post gavage and 3 weeks post gavage. Total bacterial DNA was extracted from faecal pellets and HT-specific PCR performed to determine whether HT was present or not. As a positive control DNA was also extracted from a faecal pellet harvested from a TRUC mouse. PCR products were detected on 2% agarose gel electrophoresis. Although undetectable at baseline in TRnUC mice HT DNA was detectable at the earliest time point tested (1 week) and was detectable throughout the experiment, until week 3 (Figure 46A). In contrast, HT was undetectable in mice gavaged with broth alone. These data demonstrate that following gavage with HT there was successful transmission and maintenance of gastrointestinal colonization with HT in TRnUC mice (Figure 46A).

Histological assessment of the colons of TRnUC mice newly colonized with HT demonstrated that colitis could also be transmitted following GI colonization with HT. In contrast to TRnUC mice gavaged with broth alone, TRnUC mice gavaged with HT developed severe colitis that was histologcially indistinguishable from chronic TRUC colitis (Figure 46B).

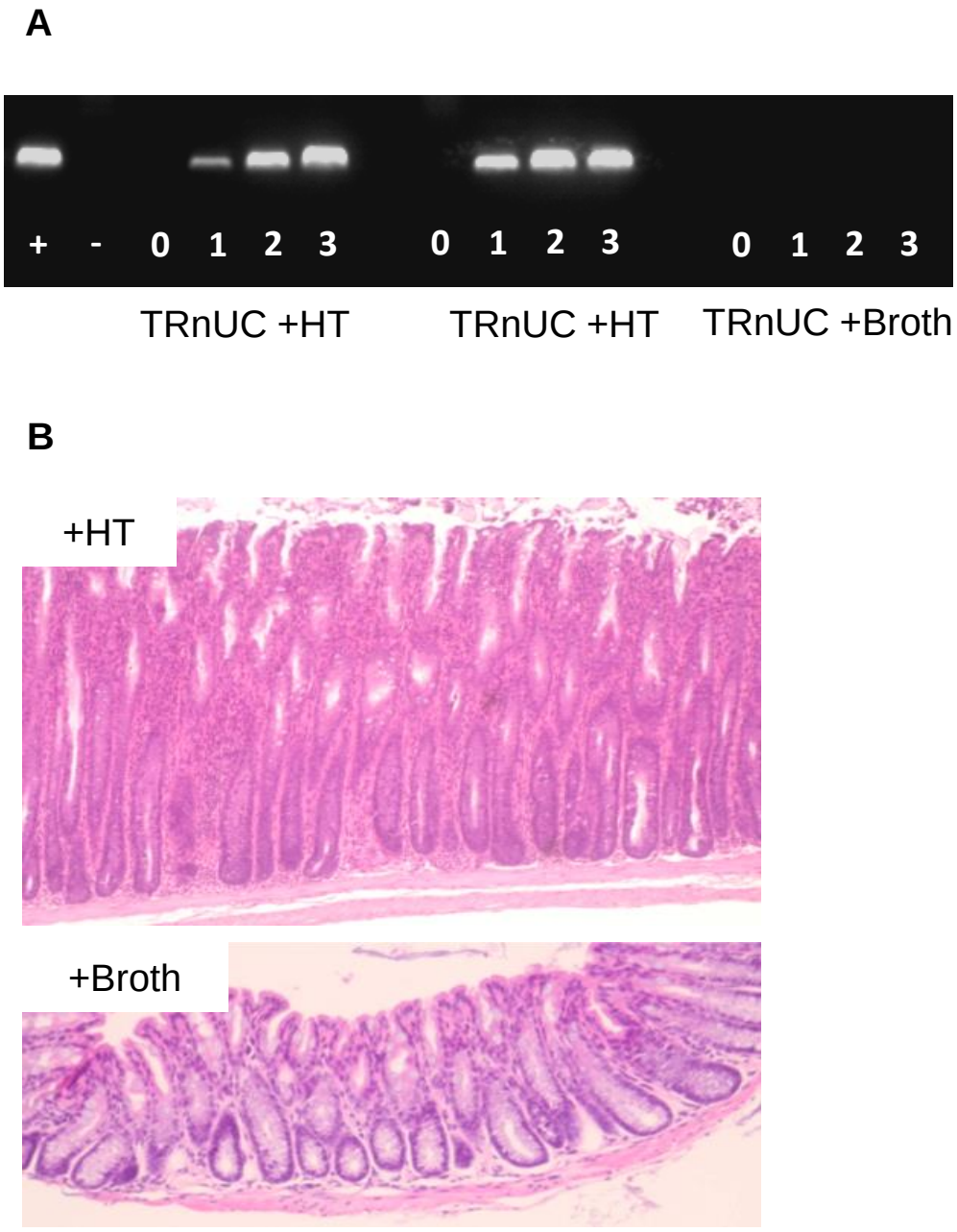


Figure 46. ***H.typhlonius*** **gavage to TRnUC mice resulted in colonization and colitis transmission.** A 2% Agarose gel electrophoresis of HT-specific PCR on bacterial DNA extracted from faecal pellets from TRnUC gavaged with HT or broth alone, at weeks 0 (baseline), and then 1-3 weeks post-gavage. B Histology (H&E) of the distal colon of TRnUC mice following gavage with HT or broth alone. Magnification is the same in both panels (approximately x40). Mice were analysed 3-4 weeks post inoculation. These data are representative of >3 individual experiments with multiple biological replicates.

5.6 *H. typhlonius* was transmissible to TRnUC or *Rag2*$^{-/-}$ mice co-housed with TRUC mice

Since the initial report describing TRUC mice reported that colitis was transmissible to other non-TRUC genotypes after co-housing (or cross-fostering) mice with TRUC mice, experiments were conducted to determine whether HT could be transmitted to otherwise disease-free mice by co-housing with TRUC mice. TRnUC and *Rag2*$^{-/-}$ mice were co-housed with TRUC mice (ratio 2:1). Faecal pellets were collected directly from the co-housed mice and bacterial DNA extracted before HT-specific PCR was performed. Intestinal colonization with HT was clearly demonstrable in TRnUC and *Rag2*$^{-/-}$ mice within the first week of co-housing and was also detectable 6 weeks later (Figure 47A).

Following co-housing, TRnUC mice also developed colitis that was histologically indistinguishable from TRUC colitis (Figure 47B). TRnUC mice co-housed with TRUC mice also developed increased colonic weight, as observed in TRUC mice, consistent with the development of colonic disease (Figure 47C). In contrast, *Rag2*$^{-/-}$ mice co-housed with TRUC mice developed only modestly enlarged colons and mild histological changes in the distal colon (Figure 47B and 47C). These data demonstrated that T-bet deficiency in the innate immune system is a critical determinant of full disease penetrance in HT associated colitis.

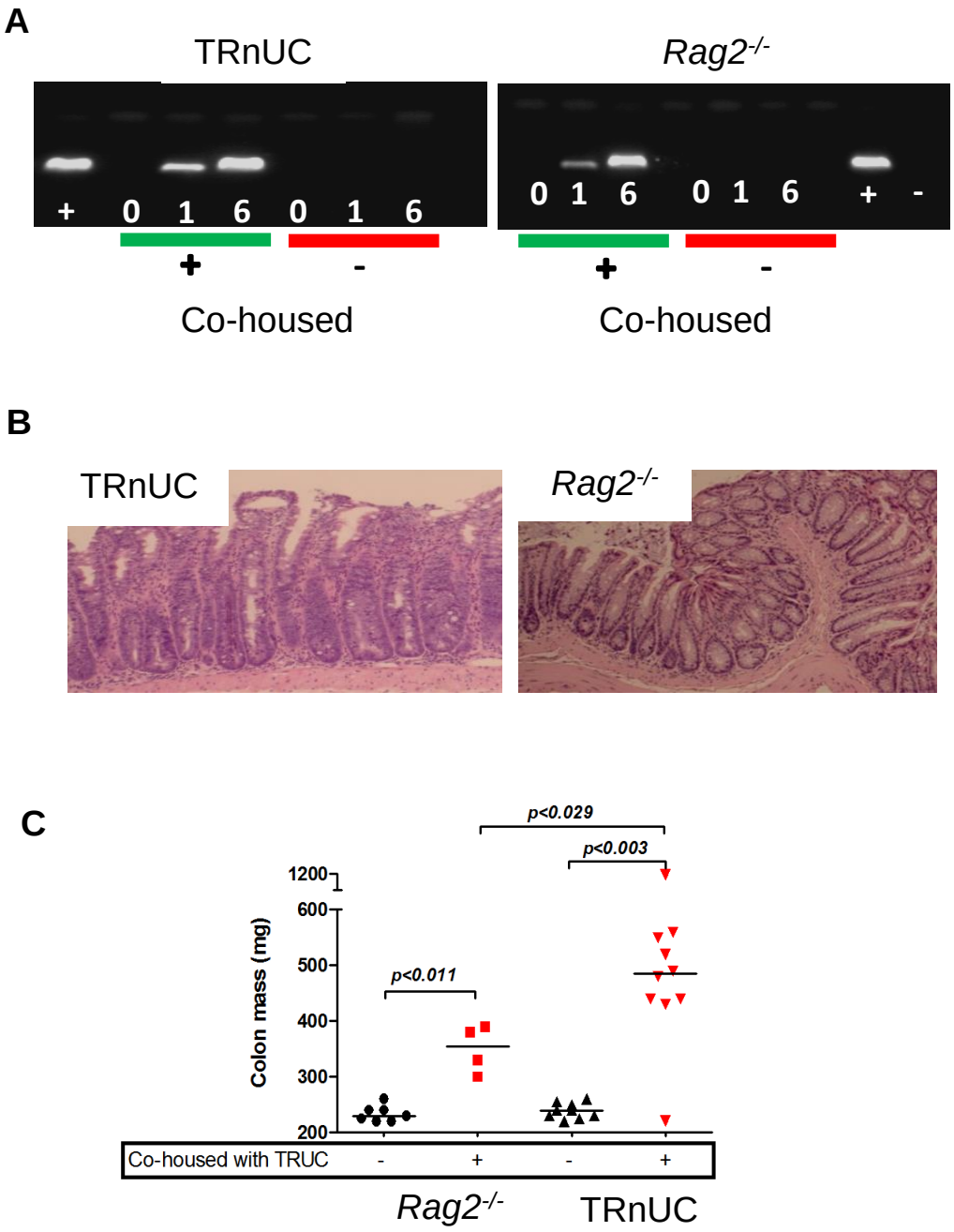


Figure 47. ***H. typhlonius*** **was transmissible to TRnUC and** ***Rag2***$^{-/-}$ **mice by co-housing with TRUC mice.** A 2% Agarose gel electrophoresis of HT-specific PCR on bacterial DNA extracted from faecal pellets from TRnUC or *Rag2*$^{-/-}$ mice co-housed with TRUC mice. Stool samples were taken at week 0 (baseline), week 1 and at 6 weeks. B Distal colon histology (H&E staining) of TRnUC and *Rag2*$^{-/-}$ mice co-housed with TRUC mice. Magnification is the same in both panels (approximately x40). C Colon mass of TRnUC or *Rag2*$^{-/-}$ mice co-housed (+) or not (-) with TRUC mice. Colon histology and disease features were analysed 6-8 weeks after co-housing was started. Each dot represents an individual mouse. Lines depict median.

5.7 *H. typhlonius* colonization induced increased TNFα transcription in the colon of TRnUC mice

Next we evaluated whether *de novo* colonization with *H. typhlonius* was capable of activating the immune pathways observed in TRUC mice. Colitis in TRUC mice is characterized by deregulated transcription of TNFα by colonic DCs (Garrett *et al*., 2007; Garrett *et al*., 2009). Therefore, TRnUC and mice were gavaged with pure cultures of HT. Following HT gavage (4-6 weeks), distal colon samples were harvested from study animals to quantify colonic transcription of *Tnfa* by qPCR.

HT inoculation to TRnUC mice resulted in a significant increase in *Tnfa* transcripts to levels seen in TRUC mice (Figure 48). Despite colonic *Tnfa* transcripts being markedly less abundant in uninfected TRnUC colons, it was noteworthy that they were significantly more abundant than in uninfected *Rag2*$^{-/-}$ colons. Together these data suggest that de-repression of the *Tnfa* locus occurs in T-bet deficient DCs independently of colitis, but by itself is insufficient to induce disease. Furthermore, HT appears to markedly augment colonic *Tnfa* transcription in the colon, particularly in mice deficient for *Tbx21*$^{-/-}$.

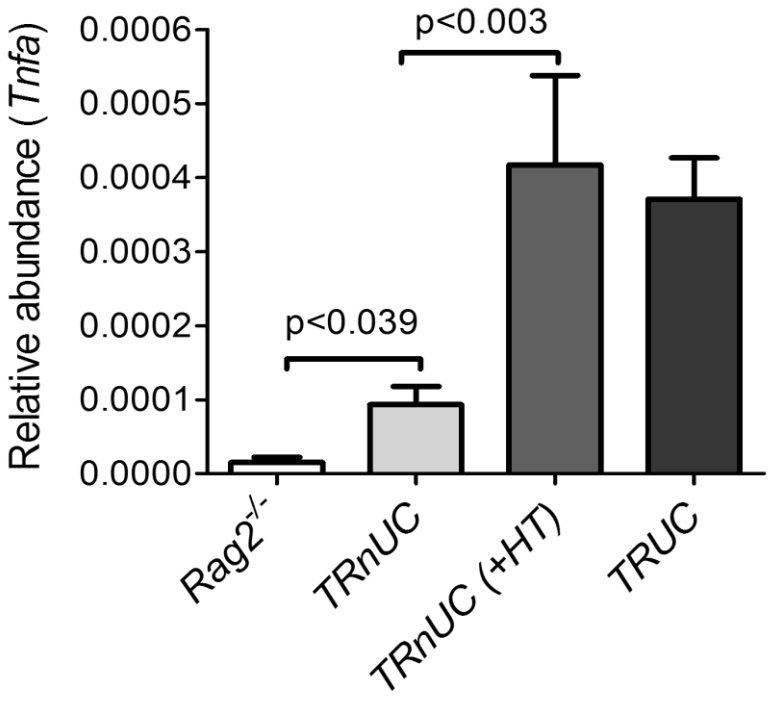


Figure 48. ***H. typhlonius*** **induced increased transcription of** ***Tnfa*** **in TRnUC mice.** qPCR for *Tnfa* in the colon of TRnUC following GI colonization with HT (+HT) or not. *Tnfa* transcripts were also quantified in the colon of *Rag2*$^{-/-}$ and TRUC mice. In this experiment *Tnfa* transcripts were quantified in the distal colon (0.5cm segment within 0.5cm of the anal verge) in 6x *Rag2*$^{-/-}$, 9x TRnUC, 4x TRnUC (post-colonization with H.T.) and 14 TRUC mice. Bars denote mean relative abundance of *Tnfa* transcripts and error bars denote SEM. These data are from a single experiment and was not repeated. Adapted from Powell *et al*., 2012.

5.8 The major colonic DC subsets were the same in TRnUC and *Rag2*$^{-/-}$ mice following HT colonization

Dysregulated TNFα production by colonic CD11c^{+} DCs is a hallmark feature of TRUC IBD (Garrett *et al*., 2007; Garrett *et al*., 2009). CD11c^{high} MHC class II^{+} intestinal DCs can be divided into subsets based on surface expression of CD103 and CD11b (Varol *et al*., 2009), with different functional properties. CD11b^{+} intestinal DCs (often co-expressing CX_3CR1) are believed to play an important inflammatory role in intestinal inflammation through production of cytokines such as TNFα (Varol *et al*., 2009). In contrast, CD103^{+} DCs imprint gut homing properties to circulating lymphocytes, and are thought to be involved in regulatory processes, including promoting FoxP3^{+} T-reg differentiation (Coombes *et al*., 2007). CD103^{+} DCs are less effective producers of inflammatory cytokines, such as TNFα, IL-6, IL-12p40 and IL-23p19 (Coombes *et al*., 2007).

One possibility for the exaggerated TNFα response observed in T-bet deficient hosts is that T-bet might alter the profiles of the different DC subsets. Indeed an imbalance in the composition of gut DC subsets, such as an inappropriate expansion of CD11b^{+} DCs, could potentially account for enhanced production of TNFα in TRUC mice. Therefore, it was determined whether the profile of intestinal DCs was altered in mice lacking T-bet in the innate immune compartment.

In the cLP of TRnUC and *Rag2*$^{-/-}$ mice DCs were defined as CD11c^{high} class II^{+} cells (Figure 49A). DCs accounted for approximately 25% of cells present. Among the CD11c^{high} classII^{+} cells, there were 3 major DC subsets seen, comprising CD11b^{+} CD103^{-} cells, CD11b^{+} CD103^{+} cells and CD11b^{-} CD103^{+} cells. In TRnUC and *Rag2*$^{-/-}$ mice, CD11b^{+} CD103^{-} cells were the most common subset in the colon, accounting for approximately 75-80% of DCs (Figure 49A and 49B). The frequency of each colonic DC subset was the same in *Rag2*$^{-/-}$ and TRnUC mice.

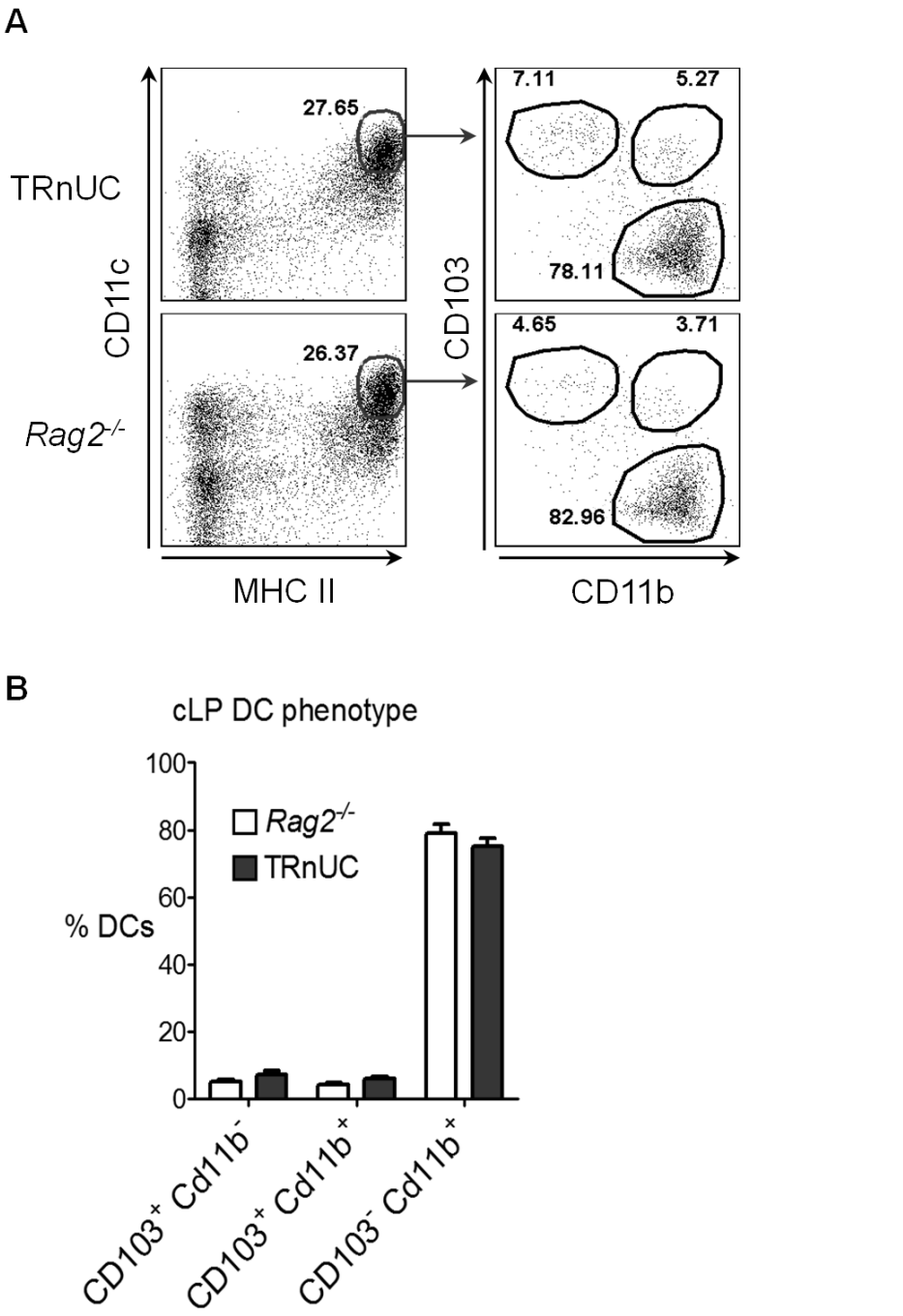


Figure 49. Colonic DC subsets in TRnUC and *Rag2*$^{-/-}$ mice. A Flow cytometry plots demonstrating colonic CD11c^{high} classII^{+} DCs (and subsets) according to CD11b and CD103 expression in TRnUC and *Rag2*$^{-/-}$ mice following inoculation with HT. B Quantification of DC subsets present in the colon of TRnUC and *Rag2*$^{-/-}$ mice 4-6 weeks following inoculation with HT. Bars denote mean proportion (%) of CD11c^{+} classII^{+} DCs of each subset. Error bars denote SEM. The relative proportion of DC subsets were defined in 5x *Rag2*$^{-/-}$ and 5x TRnUC mice. These experiments were repeated on a further 2 occasions. Adapted from Powell *et al.*, 2012.

5.9 $CD11b^{+}$ $CD103^{-}$ colonic DCs were responsible for enhanced production of TNFα in the absence of T-bet

Since the profile of colonic DC subsets was not altered in TRnUC and *Rag2*$^{-/-}$ mice, experiments were conducted to determine which DC subsets were responsible for excess TNFα production.

Notably, $CD11b^{+}$ $CD103^{-}$ DCs were the principal subset responsible for TNFα production in TRnUC and *Rag2*$^{-/-}$ mice (Figure 50A). However, there was a 2-3 fold increase in the percentage of TNFα producing cells in the $CD11b^{+}$ $CD103^{-}$ DC subset in TRnUC mice in comparison to *Rag2*$^{-/-}$ mice (Figure 50B).

Together these data show that $CD11b^{+}$ $CD103^{-}$ DCs are the principle subset responsible for excess TNFα production in the colon of HT infected mice, and that in the absence of T-bet there is a 2-3 fold increase in the percentage of $CD11b^{+}$ $CD103^{-}$ DCs that express TNFα.

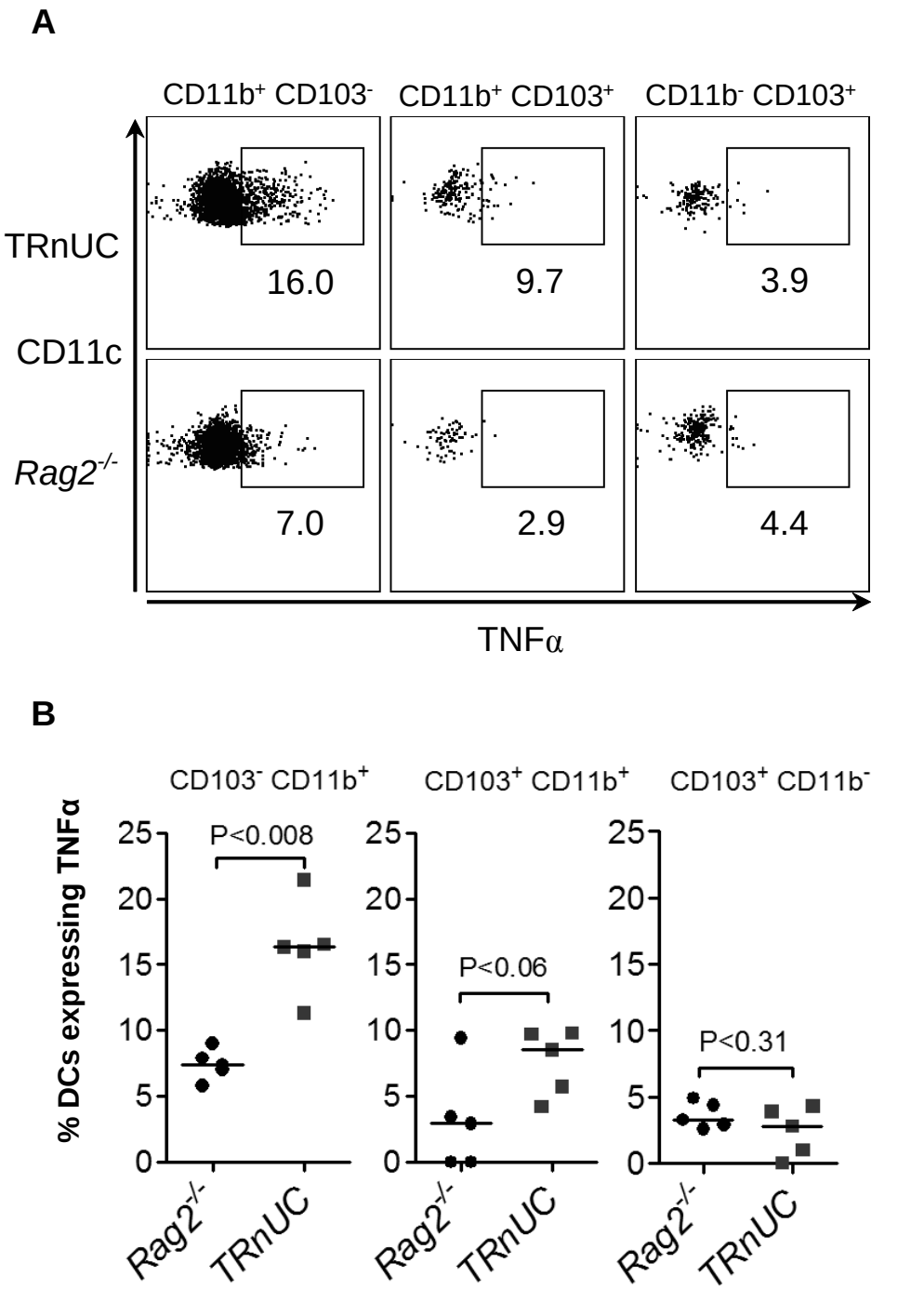


Figure 50. CD11b^{+} CD103^{-} DCs were the principle source of TNFα in TRnUC mice A Flow cytometry plots demonstrating the percentage of cells expressing intracellular TNFα expression in different CD11c^{high} classII^{+} DC subsets in the colon of TRnUC and *Rag2*$^{-/-}$ mice following inoculation with HT, and B statistical analysis (n=5 mice in each group). In this experiment unfractionated cLPLs were incubated at 37^{o}C for 4 hours in the presence of monensin without any additional exogenous stimulus. Gates were defined by incubating control cells on ice without monensin. DC subsets were defined as in Figure 49. Adapted from Powell *et al*., 2012.

5.10 TNFα potentiated IL-23 induced innate IL-17A expression in TRUC disease

Since chronic TRUC disease is dependent on ILCs, the hypothesis that TNFα produced by gut $CD103^{-}$ $CD11b^{+}$ DCs might impact on ILC activation in TRUC IBD was tested. To determine whether TNFα might influence innate IL-17A production, unfractionated mLN cells were incubated in the presence or absence of recombinant TNFα. Although on its own, recombinant TNFα was unable to induce significant innate IL-17A production, it did potentiate IL-23 induced IL-17A production (Figure 51). Indeed, the combination of TNFα and IL-23 potently and significantly increased IL-17A production in comparison with unstimulated cells or cells stimulated with IL-23 alone.

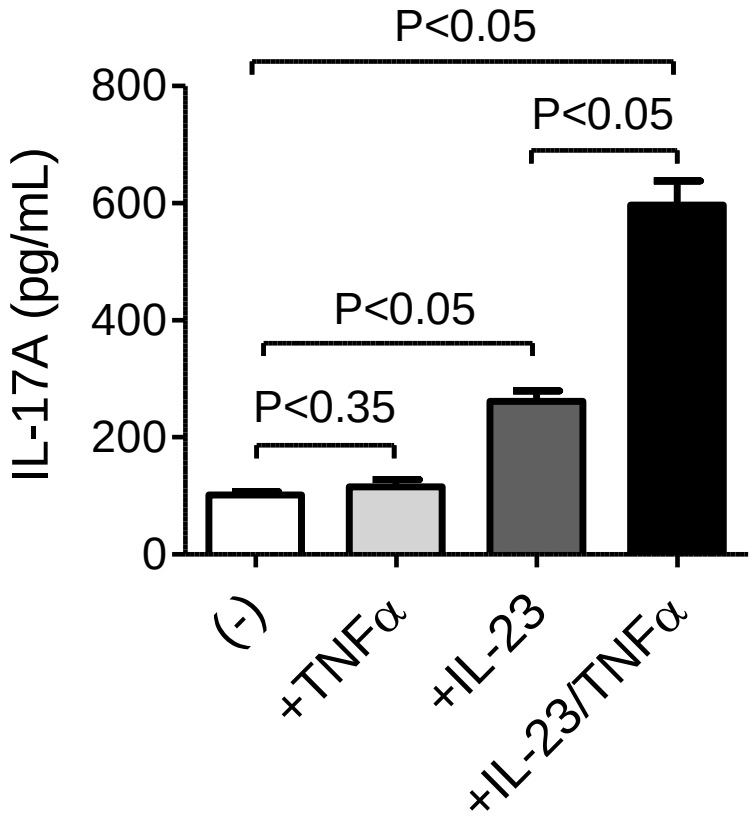


Figure 51. TNFα augmented IL-23 induced IL-17A production by ILCs from TRUC mice. Unfractionated mLN cells ($1x10^5$ cells/mL) from TRUC mice (n=5) were cultured with specified cytokines or left unstimulated (-) for 24 hours. IL-17A secreted into culture supernatants was measured by ELISA. IL-23 was used at a final concentration of 20ng/mL and TNFα was used at a concentration of 20ng/mL. Bars represent mean IL-17A concentration in culture supernatant and error bars denote SEM. These data are from a single experiment, which was not repeated. Adapted from Powell *et al*., 2012.

5.11 TNFα blockade attenuated *H. typhlonius* induced colitis

The functional importance of TNFα in HT induced colitis in TRnUC mice was also evaluated and its potential impact on ILC- derived IL-17A production.

Two weeks following inoculation with HT, TRnUC mice were treated with an *i.p.* injection (500µg) of an anti-TNFα mAb (clone XT3.11) or control isotype antibody. Mice were treated on days 0, 3, 6, 9, 12, 15 and 18, culled on day 21 and organs harvested. Anti-TNFα treatment significantly attenuated HT induced colitis in TRnUC mice with improved histological appearances (Figure 52A) and reduced colitis scores (Figure 52B).

Notably, TNFα blockade substantially reduced colonic *Il17a* transcripts, consistent with the possibility that TNFα augmented IL-17A production is physiologically relevant in HT associated ILC mediated colitis (Figure 52C).

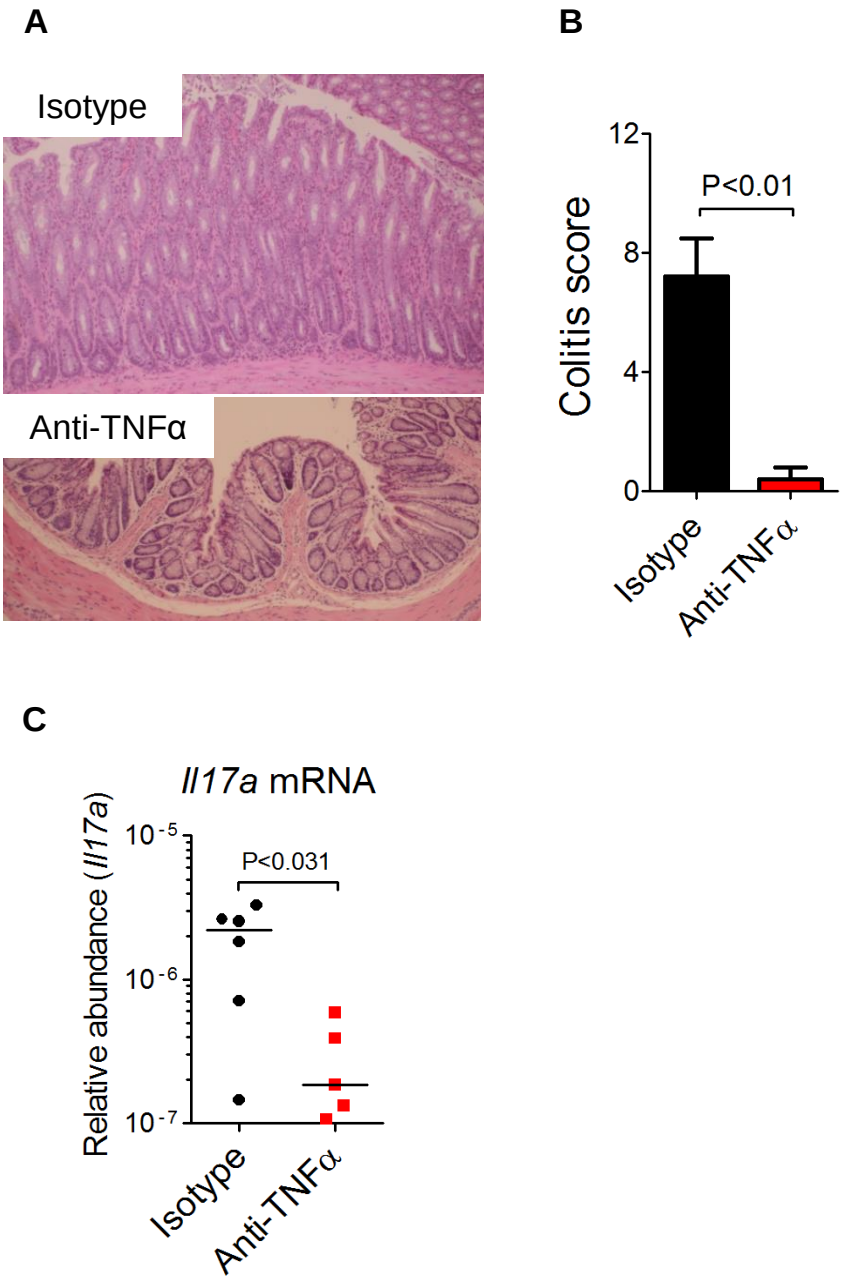


Figure 52. TNFα blockade attenuated HT associated colitis in TRnUC mice. A Distal colon histology (H&E staining) and colitis scores. Magnification is the same in both panels (approximately x40). B from TRnUC mice inoculated with HT following treatment with anti-TNFα or control isotype antibody. Bars show mean colitis score and error bars denote SEM. C qPCR of *Il17a* mRNA in the colon of TRnUC mice given HT and treated with anti-TNFα or control isotype antibody. Six mice received isotype control antibody and 5 mice received anti-TNFα antibody. Each dot represents an individual mouse. Lines depict median relative abundance of *Il17a* transcripts. Like other *in vivo* blockade experiments these data are from a single experiment which was not repeated. Adapted from Powell *et al*., 2012.

5.12 Discussion

Interactions between the host immune system and the intestinal microbiota are key determinants in maintaining intestinal homeostasis. For the host, an intact innate immune system is needed to sense luminal microbes and their products, and engage in critical decisions to generate protective, tolerogenic or inflammatory responses. In this thesis the way in which mice lacking T-bet and an adaptive immune system interact with the intestinal microbiota has been examined. In contrast to previous work this study shows that T-bet deficiency is necessary but not sufficient for the induction of colitis, as newly derived, conventionally colonized SPF *Tbx21*$^{-/-}$ *x Rag2*$^{-/-}$ mice did not develop spontaneous colitis without an additional environmental stimulus. *H. typhlonius* is now identified as the microbial constituent present in the microbiota of TRUC mice that can serve as an environmental trigger responsible for instigating colitis, particularly in *Tbx21* deficient hosts. In many ways the TRUC model of IBD can therefore be considered a useful paradigm of human IBD where the genetic architecture of the host is only really a risk factor for IBD development rather than guarantee. Indeed, in the absence of appropriate environmental cues even individuals with a "high risk" genetic background do not necessarily develop IBD. This point is exemplified by the lack of full concordance seen in IBD development in monozygotic twins (Tysk *et al*., 1988). In agreement with this there are many more healthy individuals with polymorphisms at IBD susceptibility loci than there are IBD patients.

In this study identification of *H. typhlonius* as the key pathobiont responsible for driving colitis in TRUC mice is novel and redefines the paradigm of this model of disease. It is noteworthy that the initial paper reporting the TRUC model of disease did identify *Helicobacter* present in the faeces of TRUC mice using genus generic primers (personal communication Professor Lord), however, *Helicobacter hepaticus* and *Helicobacter bilis* infection were excluded by specific PCR tests. Importantly, however, these initial analyses did not rule out the presence of *H. typhlonius*. Garrett *et al.,* also recently performed 16S rRNA gene sequencing comparing *Rag2*$^{-/-}$ and TRUC mice (Garrett *et al.,* 2010). In the supplemental materials provided online in this

paper a *Helicobacter* species was detected in the intestinal microbiota of their conventionally housed SPF mice (by 16S rRNA gene sequencing and qPCR). They state that the *Helicobacter* identified was the closely related species *Helicobacter ganmani*, but unfortunately the 16S rRNA gene DNA sequence is not provided, nor are the primer sequences for PCR amplification, therefore, it is not possible to determine whether this OTU was misclassified. Also, the *Rag2*$^{-/-}$ mice used for comparison/controls in the Garrett study are arguably not the optimal control group to employ for such analyses. It is likely that the causative bacterium in TRUC colitis was also present in the *Rag2*$^{-/-}$ controls (and therefore not seen as a unique organism to the TRUC colony), especially as these *Rag2*$^{-/-}$ controls would have been direct descendants of the same *Rag2*$^{-/-}$ colony that founded the original TRUC colony. Unfortunately the original raw sequence data from this study is not available for analysis to check for the presence of *H. typhlonius* as this dataset has not been deposited in a central sequence repository such as the NCBI Short Read Archive. In addition, these previous data only provided species-level bacterial phylotypes that were significantly different between *Rag2*$^{-/-}$ and TRUC mice (Table S1 supplemental data, Garrett *et al.*, 2010). The bacteria common to both *Rag2*$^{-/-}$ and TRUC mice are not available to evaluate. It is therefore unfortunately not possible to determine whether *H. typhlonius* was present in the intestinal microbiota of TRUC and *Rag2*$^{-/-}$ mice in that study. In contrast, isogenic newly derived *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice without colitis are a more appropriate control group. By analysing isogenic mice in the presence and absence of disease, this approach is likely to be more sensitive to define any changes in microbial composition associated with disease (and not due to alterations in host genotype). There are also technical differences in methodology that may be particularly relevant between the two studies. Garrett *et al*. used V2 region primers for amplification, whereas in this study Professor Parkhill and Dr Alan Walker used a longer stretch of the 16S rRNA gene, covering the V3-V5 regions. This may be a critically important distinction since *H. typhlonius* appears to include a rather large sequence insertion in the V2 region (Franklin *et al*., 2001). In this manuscript the authors that first described *H. typhlonius* state that "A 166-bp intervening sequence (IVS) was identified following position 198 in all [*H. typhlonius*] isolates. This IVS occupies the area normally occupied by a seven-base stem-loop centred on position 210 in several other species of *Helicobacter*.").

Therefore, at the V2 region of the 16S rRNA gene that was looked at by Garrett *et al*. it is possible that any *H. typhlonius* sequences present in their dataset would either not have aligned properly or would have been filtered out during processing as they would have falsely looked like chimeras, relative to the other sequences in their database. In contrast, since there are no insertions over the V3-V5 area of the 16S rRNA gene, it is likely that the primers used in our study would be able to detect *H. typhlonius* effectively. Indeed, in this study, the species of *Helicobacter* present was identified independently by 2 separate platforms, including DNA sequencing of the PCR product generated by *Helicobacter* genus generic primers.

H. typhlonius is a Gram negative, microaerophilic, unease negative, spiral rod-shaped bacterium. Amongst the growing number of *Helicobacter* species known to colonize laboratory mice, it is most closely related to *H. hepaticus,* with whom it shares 97.6% sequence similarity at the 16S rRNA gene locus (Franklin *et al*., 2001). In contrast to other *Helicobacter* species, including *H. hepaticus*, *H. bilis* and *H. rodentium* it does not induce hepatitis or billiard disease (Franklin *et al*., 1999), consistent with the phenotype of TRUC mice, which do not develop liver pathology (data not shown). In common with other *Helicobacter* species, *H. typhlonius* has been shown to induce colitis in mice, particularly in the context of immunodeficiency (Fox *et al*., 1999; Franklin *et al*., 1999). *H. typhlonius* is killed by desiccation, and is difficult to culture using conventional techniques.

In the study reported by Garrett it was also suggested that *Klebsiella pneumoniae* and *Proteus mirablis* may be important in colitis development following culture of these bacteria from TRUC faeces (Garrett et al., 2010). However, these organisms by themselves or in combination were unable able to induce colitis in germ free mice. In our study *Klebsiella pneumonia* was not detected in the faeces of TRUC mice by either PRIA panel testing or 454 sequencing, suggesting that this bacterium must be of very low abundance or not present at all in our TRUC colony, which would argue against a significant role for this bacterium in this model of colitis.

Identification of *H. typhlonius* as the key driver of colitis also allowed some of the immune mechanisms of TRUC disease to be interrogated. Although increased *Tnfa* transcription was observed in the absence of colitis in TRnUC mice, intestinal colonization with *H. typhlonius* was required for maximal *Tnfa* transcription and

indeed for disease induction. The disease phenotype of otherwise healthy TRnUC mice following *de novo* infection with *H. typhlonius* was clinically, histologically and immunologically indistinguishable from chronic TRUC colitis. Analysis of the DC populations in TRUC and *Rag2*$^{-/-}$ mice demonstrated that CD11b^{+} CD103^{-} DCs were both the most frequent DC subset in the colon of these mice as well of the being the subset with the greatest proportion of cells which stained positive for intracellular TNFα. Notably, the proportion of TNFα CD11b^{+} CD103^{-} DCs was significantly higher in TRnUC mice inoculated with *H. typhlonius* in comparison with *Rag2*$^{-/-}$ mice. It should be noted that the nomenclature, definitions and phenotypic markers associated with DCs often overlaps with that of other myeloid populations and in particular with tissue macrophages. In this study colonic DCs were defined in flow cytometry experiments as CD11c^{high} class II^{+} cells. It is possible that some of these cells may include colonic macrophages and therefore in some ways it might be more accurate to refer to this population as CD11c^{high} class II^{+} CD11b^{+} CD103^{-} myeloid cells. However, for consistency these cells will however be referred to as DCs, accepting the potential limitation of this definition. Irrespective of the definitions employed to define this particular population of cells in mice lacking T-bet there is dysregulated/increased expression of TNFα in comparison with the analogous population of cells in T-bet sufficient hosts. Additional support for the likelihood that this cell population is a DC population is that T-bet is expressed by DCs (Lighvani *et al*., 2001), but its expression has never been described in macrophages. These data also support data from other groups indicating that CD11b^{+} CD103^{-} DCs (or myeloid cells) are primarily "inflammatory" in nature (Varol *et al*., 2009).

In keeping with previous observations, colitis was also transmissible to T-bet sufficient *Rag2*$^{-/-}$ hosts, albeit with markedly diminished severity in comparison with *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ mice. These data highlight how host genotype profoundly influences the cross talk between the mucosal immune system and particular components of the intestinal microbiota. Despite the presence of a diverse community of microbes, the addition of a specific microbe, in this case *H. typhlonius* is required to exploit the colitogenic susceptibility of mice lacking T-bet in the innate immune system. Data are now beginning to emerge to suggest that particular intestinal microbes might also influence intestinal homeostasis in humans. Adherent invasive *Escherichia coli* has been implicated in the pathogenesis of Crohn's disease

(Chassaing *et al*., 2011) and norovirus infection, an acute gastroenteritis in humans, has also recently been identified as a key disease trigger in a mouse model of Crohn's disease (Cadwell *et al*., 2010), although notably, MNV was absent from TRUC and TRnUC colony members in this study (PRIA panel screening). Conversely, the presence of *Faecalibacterium prausnitzii* appears to be negatively correlated with IBD (Joossens *et al*., 2011; Sokol *et al*., 2008). These data suggest that under certain circumstances specific microbes may be capable of exploiting the genetic susceptibility of IBD prone individuals.

In conclusion, T-bet deficiency is insufficient to cause colitis by itself, but additional environmental signals are required to trigger disease. It is now clear that particular intestinal microbes are necessary to exploit this susceptibility, and the proteobacterial species *H. typhlonius* has been identified as the key environmental stimulus responsible for precipitating IBD in TRUC mice. These findings in the mouse mirror some of the current thinking regarding the likely pathogenesis of human IBD, where it is now thought disease is probably due to exposure of a genetically susceptible individual to an appropriate environmental trigger.

CHAPTER 6

Results: Defining the mechanisms whereby T-bet controls colitogenic immune pathways in TRUC mice

T-bet was first described as the master transcription factor responsible for controlling the phenotype of $CD4^+$ Th1 T-cells (Szabo *et al*., 2000). T-bet binds at the *Ifng* locus, where it transactivates the expression of this gene and is required for optimal production of IFNγ by Th1 cells (Szabo *et al*., 2000). $CD4^+$ T-cells from *Tbx21*$^{-/-}$ mice not only have severely impaired IFNγ production, but also have a default gain of function with respect to their differentiation towards other effector lineages, such as Th17 cells (Lazarevic et al., 2011). In view of the dominant role played by T-bet in controlling the profile of cytokines produced by T-cells, the hypothesis that T-bet would also control the profile of cytokine produced by ILCs was also tested. In addition, existing data from the laboratory, including chromatin immunoprecipitation (ChIP) data from fellow students were analysed to determine whether other ILC-related genes might be impacted by T-bet (or its genetic deficiency) in TRUC mice.

6.1 T-bet deficient ILCs were relatively poor producers of IFNγ and instead preferentially expressed IL-17A in *H. typhlonius* associated colitis

In the first results Chapter it was observed that ILCs from TRUC mice exhibit a highly polarized IL-17A response and lack significant IFNγ expression, suggestive of the possibility that T-bet is also required for optimal IFNγ production by innate immune cells in TRUC disease. To test this hypothesis, the phenotype of *Tbx21*$^{+/+}$ and *Tbx21*$^{-/-}$ intestinal ILCs was compared in the context of *de novo* intestinal colonization with HT.

TRnUC and *Rag2*$^{-/-}$ mice were gavaged with HT. Mice were culled 6 weeks later. Colonic histology revealed that TRnUC mice developed severe colitis and *Rag2*$^{-/-}$ mice developed minor inflammatory changes, as reflected by the colitis scores (Figure 53A). There was also a tendency for increased colon mass (Figure 53B) and

spleen mass (Figure 53C) in TRnUC recipients of HT, although this did not achieve statistical significance.

Cytokine production was studied in *ex vivo* colon explant cultures. Notably, there were significantly higher concentrations of IFNγ in culture supernatants from *Rag2*$^{-/-}$ mice in comparison with TRnUC mice ($P<0.012$, Figure 54A). Conversely, explant culture supernatants from TRnUC mice had significantly higher concentrations of IL-17A ($P<0.012$, Figure 54B).

Cytokine production was also analysed in colonic ILCs from *Rag2*$^{-/-}$ and TRnUC mice colonized with HT by flow cytometry following stimulation of unfractionated cLPLs with PMA and ionomycin. In contrast to CD90^{high} cLP cells from *Rag2*$^{-/-}$ mice where there were very few IL-17A^{+} cells seen, CD90^{high} cLP cells from TRnUC mice were a rich source of IL-17A. In this experiment there was relatively few IFNγ^{+} ILCs seen in either genotype (Figure 54C).

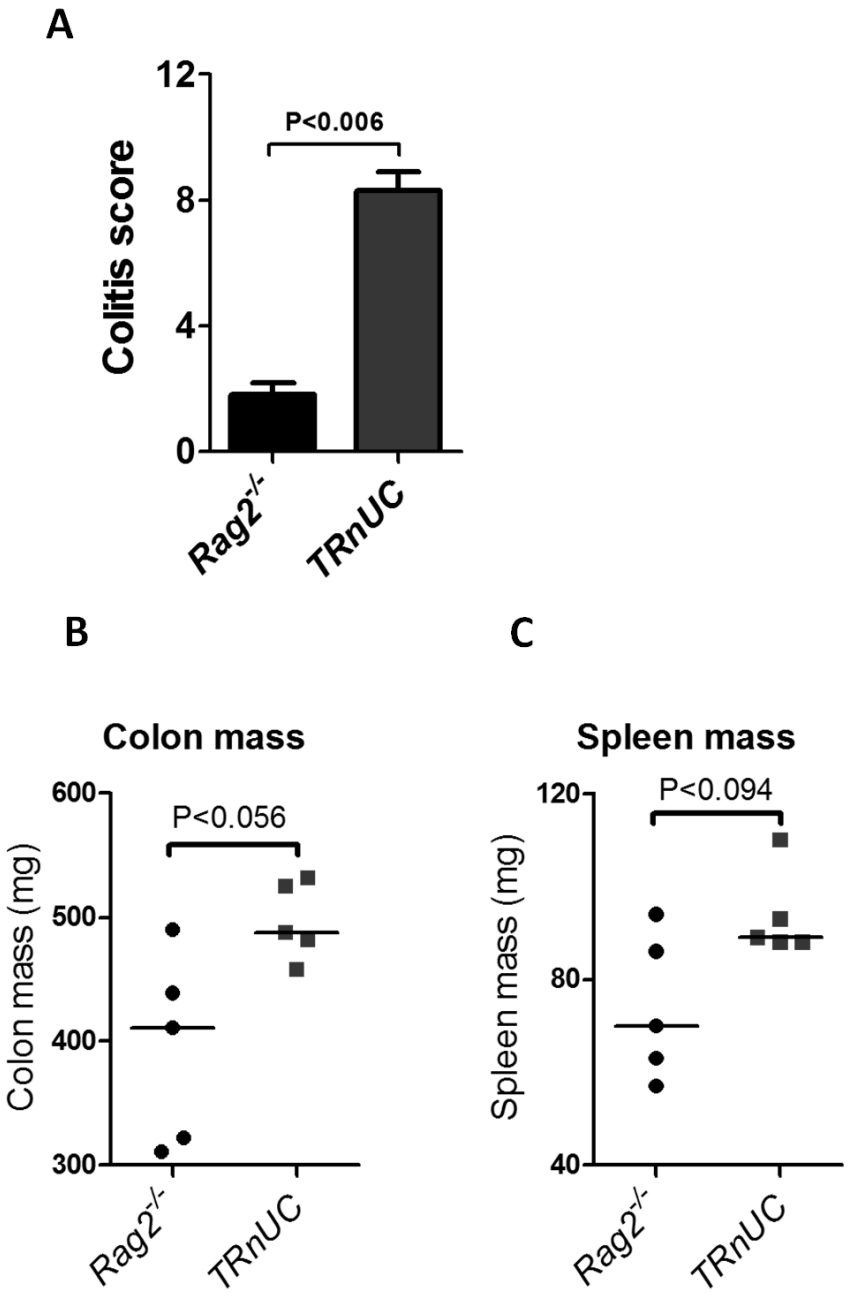


Figure 53. HT inoculation resulted in more severe colitis in TRnUC mice in comparison with *Rag2*$^{-/-}$ mice. A Distal colitis histology scores, B colon mass and C spleen mass in *Rag2*$^{-/-}$ and TRnUC mice inoculated with HT. Five mice were used in each group. In panel A the bars represent mean colitis score and error bars denote SEM. These data are representative of an additional experiment which replicated these findings (although was performed in mice of different ages, therefore, data was not aggregated). Mice were examined 6 weeks post HT inoculation. In Figures B and C each dot represents an individual mouse. Lines depicts median of the variable defined by the y-axis.

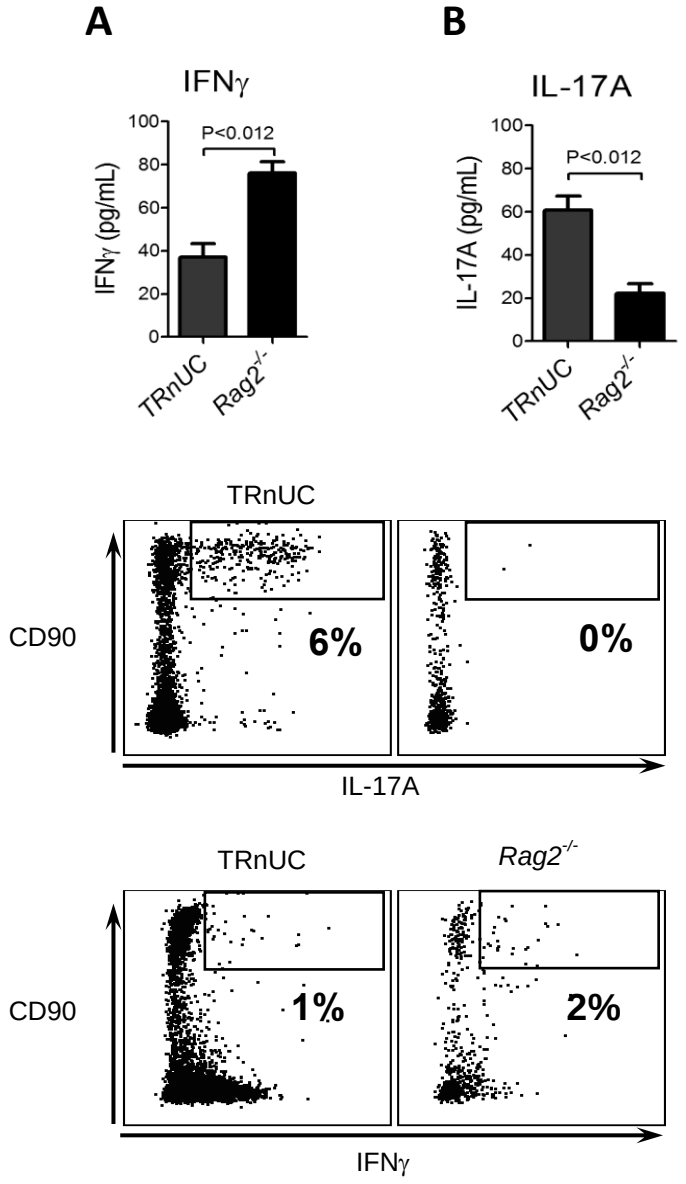


Figure 54. HT colonized mice lacking T-bet in the innate immune compartment were poor producers of IFNγ and instead preferentially produce IL-17A. A IFNγ and B IL-17A concentrations in colon explant culture supernatants from TRnUC (n=5) and *Rag2*$^{-/-}$ (n=5) mice following colonization with HT (after 4-6 weeks), measured by ELISA. Explants were cultured for 24 hours. Bars represent mean cytokine concentration in culture supernatants and error bars denote SEM. C Flow cytometry plots showing intracellular cytokine expression in the colon of TRnUC and *Rag2*$^{-/-}$ mice (colonized with HT) following stimulation with PMA and ionomycin. Cells were stimulated for 4 hours with PMA and ionomycin and in the final 2 hours monensin was added. Data are representative of multiple (>3) biological replicates.

6.2 Establishment of an additional model of ILC activation *in vivo*

ILCs can also be activated *in vivo* in lymphopaenic mice by administration of agonistic anti-CD40 mAbs (Buonocore *et al*., 2010). Therefore, an attempt was made to set up this model of ILC activation, in order to evaluate ILCs in TRnUC and *Rag2*$^{-/-}$ mice outside of the context of microbial-driven disease. This was considered necessary because some of differences observed in ILCs in the context of HT-associated colitis might have been due to the disproportionately severe disease seen in TRnUC mice in comparison with *Rag2*$^{-/-}$ mice. It has previously been shown that the anti-CD40 model of colitis occurs independently of the intestinal microbiota (Uhlig *et al*., 2006). Therefore, this model was used to further investigate the phenotype of ILCs in the presence or absence of T-bet.

An agonistic anti-CD40 mAb (125µg, clone FGK4.5) or control isotype antibody (rat IgG2a, clone 2AE) was administered (*i.p.)* to *Rag2*$^{-/-}$ mice. Mice receiving agonistic anti-CD40 mAbs rapidly started losing weight, which was statistically significant at just 1 day ($P<0.007$, Figure 55A). Mice were culled on either day 7 or 8. Abdominal dissection revealed splenomegaly, mesenteric lymphadenopathy and increased colon size (Figure 55B). Spleen weight was significantly higher in anti-CD40 treated mice in comparison with control mice ($P<0.0001$, Figure 55C). Likewise, liver weight was also significantly increased in anti-CD40 treated mice in comparison with control mice ($P<0.0001$, Figure 55D), demonstrating that *in vivo* activation of CD40 in lymphopaenic mice results in wasting and multi-system disease. Colitis changes were typically patchy, heterogeneous and very difficult to detect consistently in equivalent anatomical locations across groups of mice (data not shown). However, colon mass was reproducibly and significantly greater in anti-CD40 treated mice in comparison with isotype control treated mice ($P<0.0001$, Figure 56A). Furthermore, analysis of cLP cells demonstrated that there was marked expansion of Gr-1^{high} granulocytes in the colon of *Rag2*$^{-/-}$ mice treated with anti-CD40 (Figure 56B), consistent with induction of acute colonic inflammation in these mice.

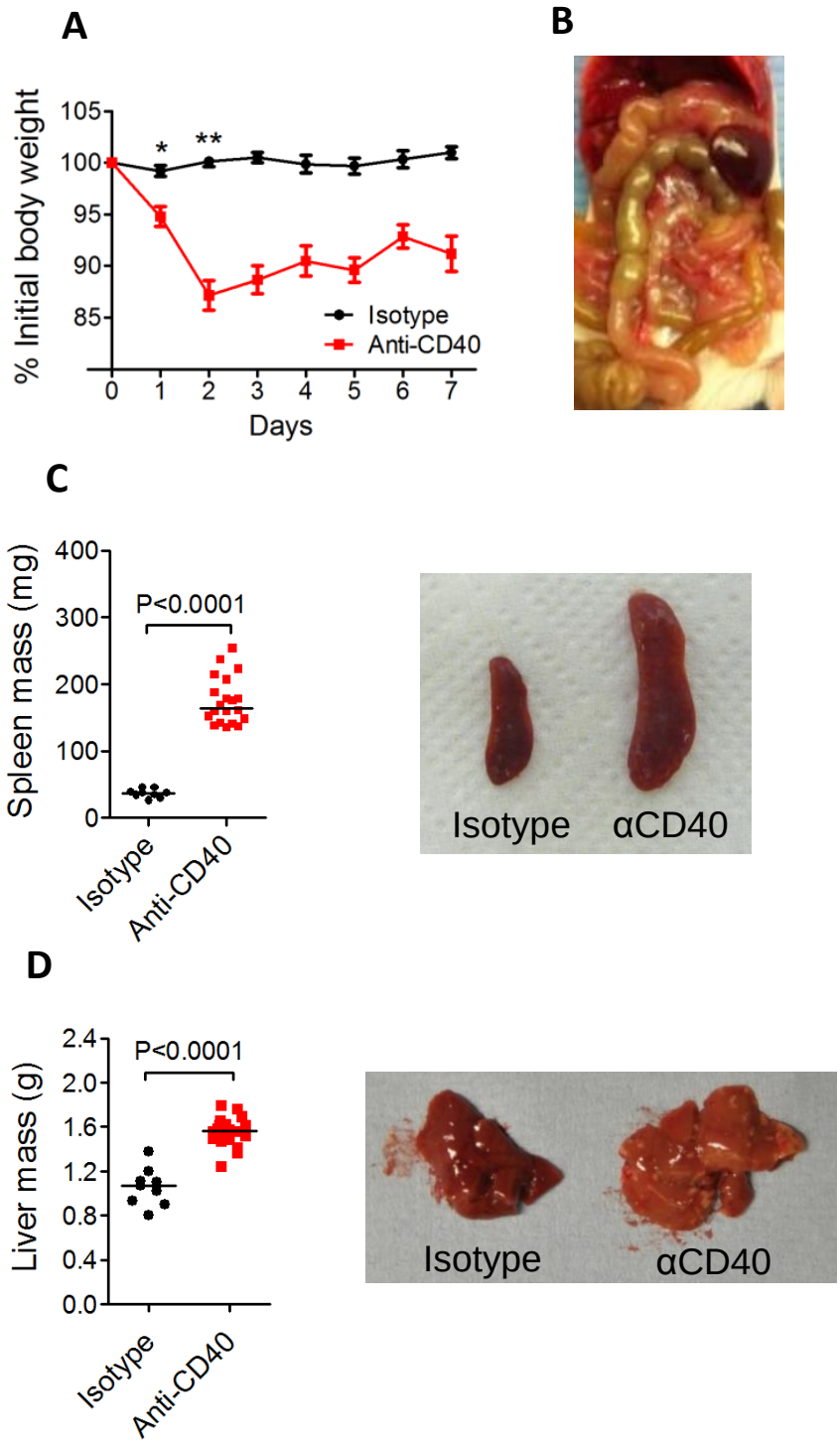


Figure 55 **Agonistic anti-CD40 mAbs induced multisystem disease in *Rag2*$^{-/-}$ mice.** A Body weight (as a % of initial body mass) in *Rag2*$^{-/-}$ mice treated with anti-CD40 mAb (n=7) or control isotype (n=6). *P<0.007, **P<0.002. B Abdominal dissection of *Rag2*$^{-/-}$ mouse treated with anti-CD40 mAb. Data points represent median and error bars denote SEM. Other clinical features in *Rag2*$^{-/-}$ mice treated with anti-CD40 mAb or control isotype, included changes in C spleen mass and D liver mass. In C and D each dot represents an individual mouse. Lines depicts median of the variable defined in the y-axis. Mice were examined on days 7 or 8 following anti-CD40 administration. Adapted from Powell *et al*., 2012.

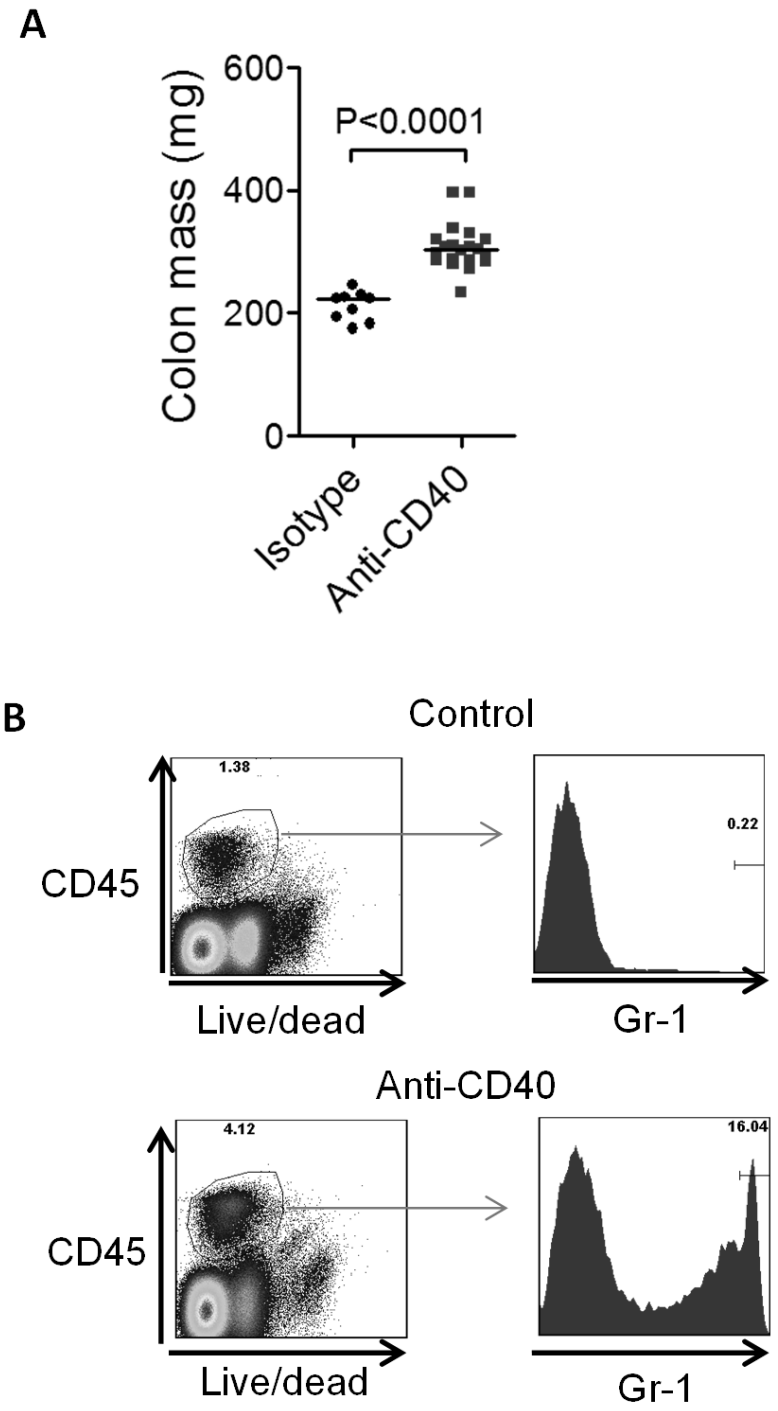


Figure 56. Agonistic anti-CD40 mAbs induced increased colonic mass and infiltration of the colon with Gr-1^{high} neutrophils. A Colon mass in *Rag2*$^{-/-}$ mice treated with anti-CD40 mAb or control isotype. Each dot represents an individual mouse. Lines depict median mass. B Flow cytometry plot showing the proportion of Gr-1high cells in the live, CD45^{+} gate in *Rag2*$^{-/-}$ mice treated with anti-CD40 mAb. These data are representative of multiple different biological replicates. These experiments were repeated multiple (>3) times with multiple biological replicates. Mice were examined on days 7 or 8 following anti-CD40 administration. Adapted from Powell *et al*., 2012.

6.3 CD90^{+} cells in *Rag2*$^{-/-}$ mice expressed T-bet

If T-bet significantly impacts ILC effector function, showing that T-bet protein is expressed by gut ILCs would provide supportive evidence. Therefore, the expression of T-bet in ILCs was investigated in *Rag2*$^{-/-}$ mice following induction of disease with anti-CD40. TRnUC mice were used as controls.

Flow cytometry experiments were performed to assess T-bet and cytokine expression in intestinal ILCs from *Rag2*$^{-/-}$ mice following innate immune activation with anti-CD40. Unfractionated cLP cells were stimulated with PMA and ionomycin. There was an appreciable IFNγ response detected in these mice, predominantly from CD90^{+} cells, although there was little IL-17A production (Figure 57A).

T-bet expression was also determined in CD90^{high} cells from *Rag2*$^{-/-}$ mice (and in TRnUC mice as controls) following activation of the innate immune system with anti-CD40. Importantly, there was clear intracellular expression of T-bet in CD90^{high} ILCs from the mLN of *Rag2*$^{-/-}$ mice. As expected there was no detectable T-bet found in ILCs from TRnUC mice (Figure 57B). These data show for the first time that T-bet protein is expressed by ILCs.

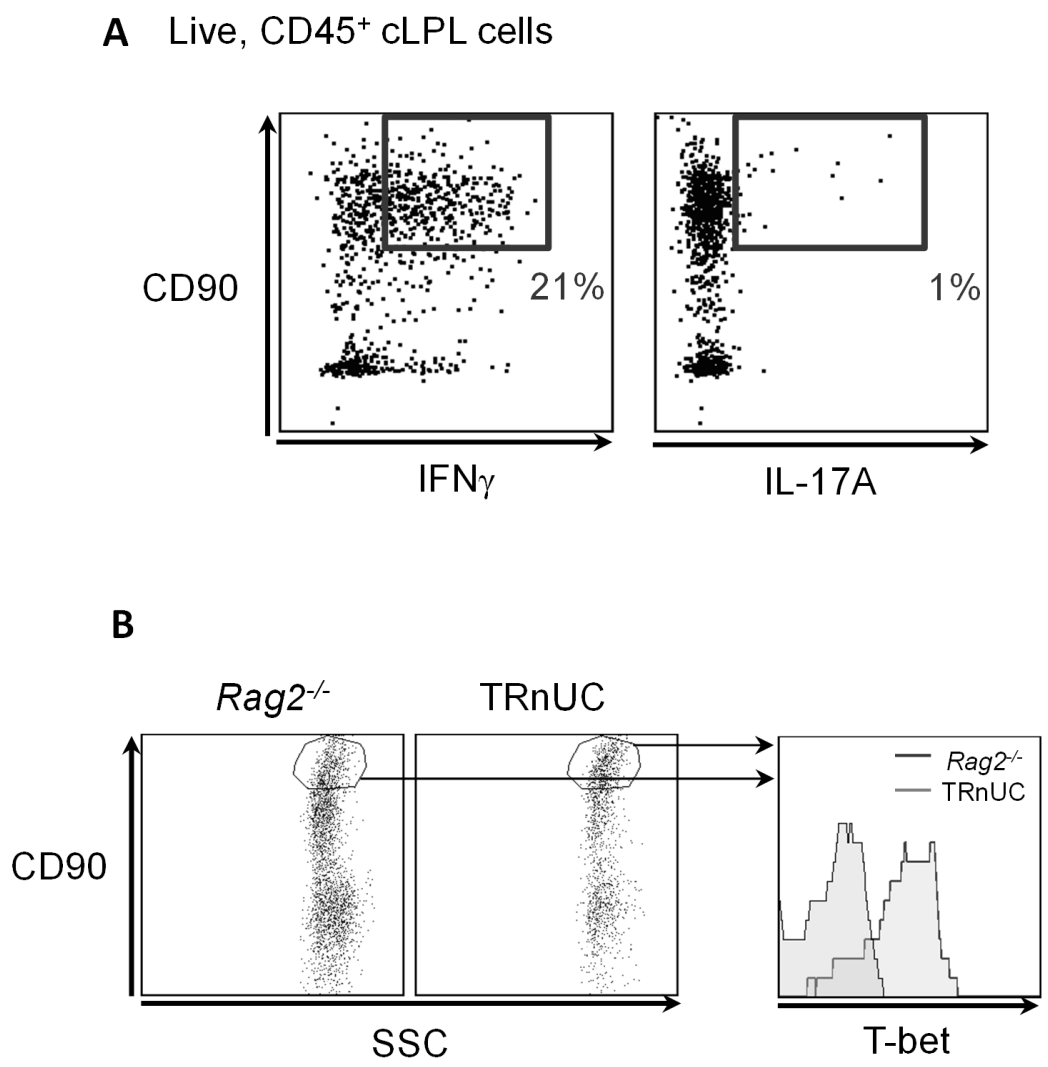


Figure 57. Cytokine and T-bet expression in the colon of *Rag2*$^{-/-}$ mice treated with agonistic anti-CD40 mAbs. A Flow cytometry plot showing CD90, IFNγ and IL-17A expression in unfractionated cLPLs from *Rag2*$^{-/-}$ mice following *in vivo* administration of anti-CD40 (cells were activated with PMA and ionomycin). B Flow cytometry histogram showing T-bet expression in CD90^{high} cells in the mLN of *Rag2*$^{-/-}$ mice following anti-CD40 administration. Anti-CD40 treated TRnUC mice were used as controls to accurately define the negative T-bet gate. These data are representative of multiple biological replicates. Experiments were also repeated on at least 3 separate occasions. Mice were examined on days 7 or 8 following anti-CD40 administration.

6.4 T-bet expression correlated with the expression of interferon-γ and was negatively correlated with IL-17A expression by intestinal ILCs in anti-CD40 induced disease

To determine how T-bet relates to cytokine expression in ILCs, the innate immune system of *Rag2*$^{-/-}$ and TRnUC mice was activated by administering anti-CD40 mAbs. Cytokine production and T-bet expression in ILCs in this disease setting was then determined by flow cytometry. mLNs were harvested from *Rag2*$^{-/-}$ and TRnUC mice following induction of disease. Unfractionated mLN cells were stimulated with PMA and ionomycin. ILCs were identified by gating live, CD90^{high} cells.

ILCs isolated from the mLN of *Rag2*$^{-/-}$ mice expressed T-bet, which positively correlated with IFNγ expression, but was inversely correlated with IL-17A expression. Conversely, IFNγ expression was markedly diminished in ILCs from TRnUC mice and instead these cells predominantly produced IL-17A (Figure 58A). The proportion of IL-17A and IFNγ positive CD90^{+} cells in the cLP was also calculated. ILCs from *Rag2*$^{-/-}$ mice were significantly more likely to be positive for IFNγ ($P<0.03$) in comparison with ILCs from TRnUC mice, whereas ILCs from TRnUC mice were significantly more likely to be positive for IL-17A ($P<0.002$) in comparison with ILCs from *Rag2*$^{-/-}$ mice (Figure 58B).

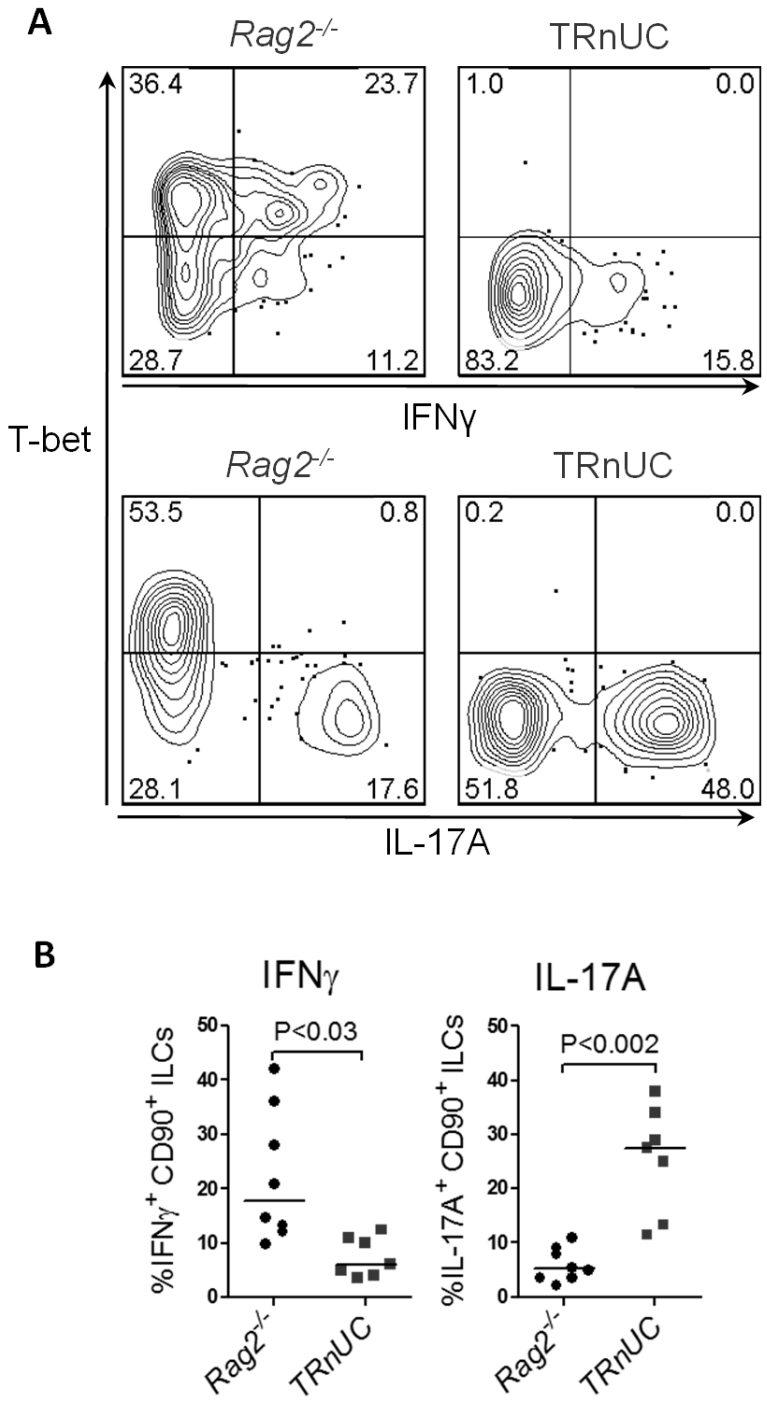


Figure 58. T-bet correlated with IFNγ and is inversely correlated with IL-17A in ILCs. A Flow cytometry plot showing intracellular cytokine and T-bet expression in ILCs ($CD90^{+}$) cells from mLN of *Rag2*$^{-/-}$ and TRnUC mice following administration of agonistic anti-CD40. These data are representative of multiple experiments (>10 individual mice). B % cytokine positive $CD90^{high}$ cells in the cLP of *Rag2*$^{-/-}$ (n=8) and TRnUC (n=7)mice following administration of agonistic anti-CD40. Mice were examined on days 7 or 8 following anti-CD40 administration. Individual dots represent individual mice and the lines depict median % of cytokine positive cells. Adapted from Powell *et al*., 2012.

6.5 Delayed wasting disease in T-bet deficient hosts

Following induction of disease with anti-CD40 mAbs, there were also differences in the disease phenotype of *Rag2*$^{-/-}$ and TRnUC mice. *Rag2*$^{-/-}$ mice lost weight significantly faster than TRnUC mice (expressed as a % of initial body weight). As early as 1 day following administration of anti-CD40, *Rag2*$^{-/-}$ mice had lost significantly more weight than TRnUC mice ($P<0.0005$, Figure 59A). In contrast, administration of anti-CD40 antibody induced a significant increase in colon weight in both of *Rag2*$^{-/-}$ and TRnUC mice, which did not differ significantly between the genotypes (Figure 59B). Colon histology was not available for this experiment, since in our hands histological changes were very patchy in this model and if anatomically consistent sections of colon (e.g. distal 0.5cm) were taken, some clearly diseased animals (i.e. with evidence of increased colon mass, weight loss and Gr-1^{high} neutrophilic infiltration of colon as assessed by flow cytometry) might have absence of clear inflammation in particular segments.

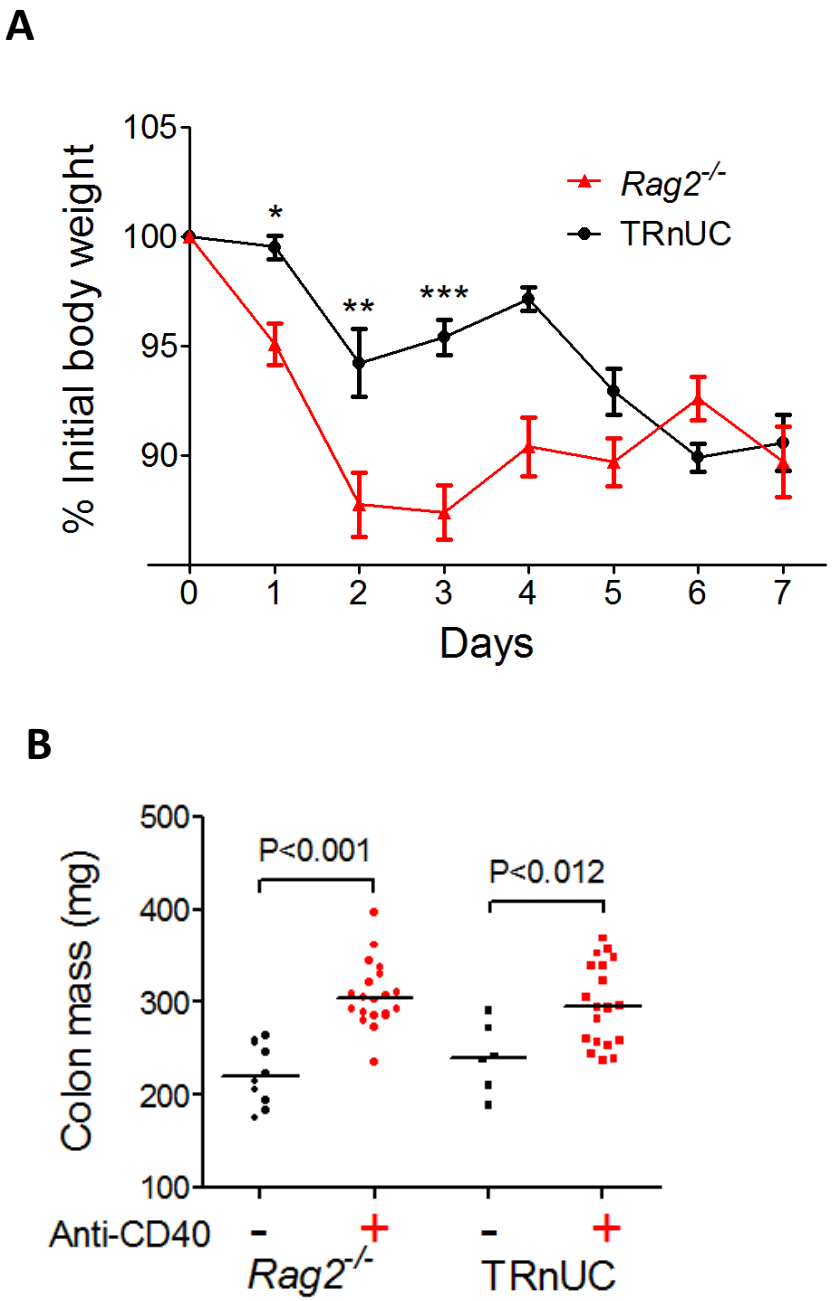


Figure 59. Distinct disease phenotypes in TRnUC and *Rag2*$^{-/-}$ mice following administration of anti-CD40 mAbs. A Weight loss (as a % of initial body weight) and B colon mass in *Rag2*$^{-/-}$ and TRnUC mice following administration of agonistic anti-CD40. *P<0.0005, **P<0.01. ***P<0.001. These data are a composite of 2 separate experiments. These experiments have been additionally repeated on at least 4 occasions with comparable results. Mice were examined on days 7 or 8 following anti-CD40 administration. Note – control antibody treated mice did not lose/gain weight in this short time span experiment (7 days). Therefore, data regarding control m ice weights are not shown to avoid overloading the graphical representation with data. In A dots represent mean weight loss and error bars represent SEM. In B each dot represents an individual mouse. Lines depict median of the variable defined in the y-axis. Adapted from Powell *et al*., 2012.

6.6 T-bet bound at the *Il7r* locus in CD4$^+$ T-cells

In Th1 cells T-bet binds at the loci encoding multiple different Th1 related molecules, to ensure that a co-ordinated, appropriately polarised response ensues. Therefore, in addition to altering the profile of cytokines produced by ILCs, it was postulated that T-bet might regulate other genes involved in ILC biology. The majority of ILCs in TRUC colitis and other ILC associated diseases (Buonocore *et al*., 2010; Sonnenberg *et al*., 2011) express IL-7R. Signalling through IL-7R has been shown to play an important role in the homeostasis of several immune cell populations (Crellin *et al*., 2010; Satoh-Takayama *et al*., 2010; Schmutz *et al*., 2009). Although ILCs are too infrequent to perform chromatin immunoprecipitation sequencing (ChIP-seq) to identify which genes are bound by T-bet, there was already data available in the laboratory regarding which genes that T-bet binds at in CD4$^+$ T-cells (Dr Refik Gokmen PhD thesis 2012, King's College London). ChIP-seq data generated by Dr Gokmen was examined to determine whether T-bet is likely to bind to the *Il7r* locus. The methodology for this experiment has subsequently been published (Gokmen *et al*., 2013). In short, naive (CD25$^-$ CD62L^{high} CD44^{low}) CD4$^+$ T-cells were sorted from unfractionated splenocytes from WT and *Tbx21*$^{-/-}$ Balb/C mice and differentiated *in vitro* with skewing cytokines (IL-2, IL-12 and anti-IL-4) into Th1 cells. Proteins were cross-linked to DNA and then immunoprecipitated with a T-bet specific antibody. DNA sequences associated with T-bet were then sequenced. T-bet binding was enriched at *Il7ra* gene locus. Importantly, binding was found at the transcriptional start site of the gene in a region highly conserved in mammals (Figure 60), consistent with the likelihood that this observation was biologically relevant. The negative control cell population used in this experiment were Th1 cells from *Tbx21*$^{-/-}$ mice, in which there was no binding of T-bet detected at the *Il7r* locus, as expected, indicating that the signals observed in T-bet sufficient Th1 cells was specific binding (Figure 60). To serve as positive control for T-bet binding the *Ifng* locus was also analysed, which also showed T-bet binding at the transcriptional start site of this gene (Figure 60). No binding was seen in *Tbx21*$^{-/-}$ Th1 cells. Comparison of T-bet binding at the *Ifng* and *Il7r* loci shows stronger binding of T-bet at the *Ifng* locus, albeit it with comparable orders of magnitude. Using model based analysis of ChIP-seq (MACS) (Zhang et al., 2008) the binding of T-bet at the

Il7r locus was identified as statistically significant binding ($P<1.0 \times 10^{-6}$), consistent with this being a true transcriptional target of T-bet.

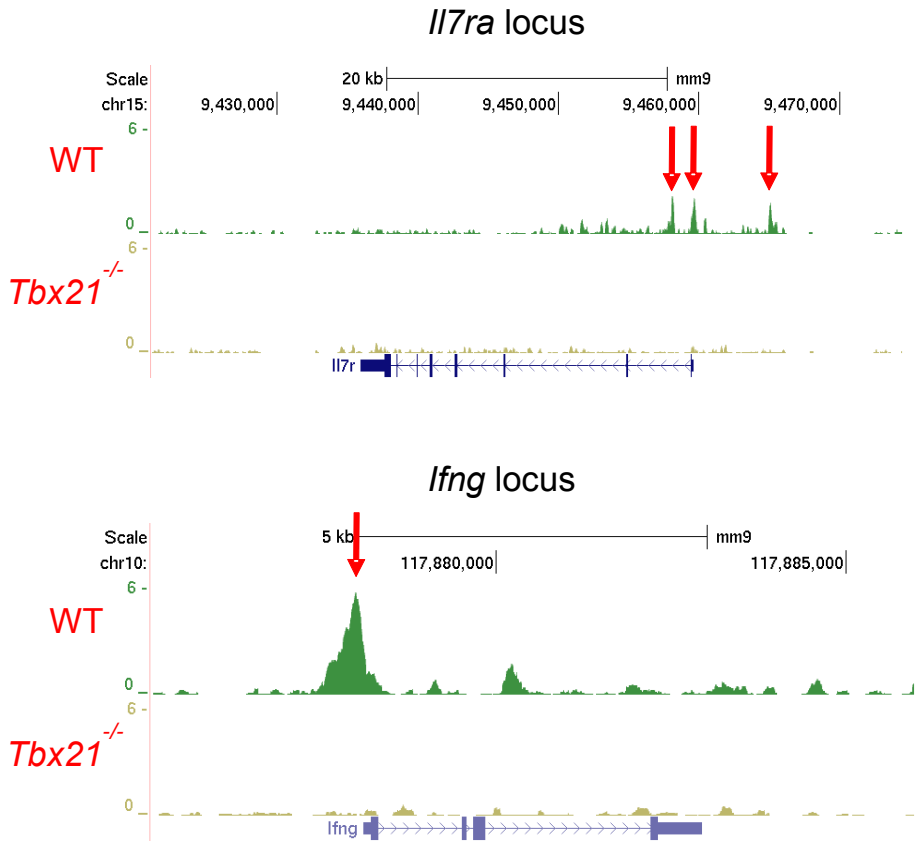


Figure 60. T-bet bound at the promoter of the *Il7r* locus in CD4$^+$ Th1 T-cells. UCSC genome browser image is shown for T-bet binding at the *il7ra* locus in *Tbx21*$^{+/+}$ vs. *Tbx21*$^{-/-}$ CD4$^+$ Th1 cells (upper panel) following stimulation with PMA and ionomycin for 4 hours. The lower panel shows T-bet binding at the *Ifng* locus, which serves as a positive control. Chromatin was immunoprecipitated, cross-lined and prepared as in Jenner et al., 2009. Samples were sequenced using an Illumina Genome Analyzer II-x. The vertical axis depicts the number of tags per million total sequences, with the genomic location running along the horizontal axis. The direction of transcription is indicated by arrows. Three T-bet binding sites are illustrated (red arrows). Whole genome data were generated by Dr R. Gökmen. Adapted from Powell *et al*., 2012.

6.7 T-bet was a transcriptional repressor of *Il7r*

To determine whether T-bet was likely to be a transcriptional repressor of *Il7ra* in ILCs, these cells were purified from the mLN of TRnUC and *Rag2*$^{-/-}$ mice following *in vivo* activation of these cells with anti-CD40. Unfractionated mLN cells were labelled with monoclonal antibodies directed against CD90 and NKp46. CD90^{high} NKp46^{-} cells were then FACs sorted and snap frozen in Trizol reagent pending cDNA synthesis and qPCR to quantify *Il7ra* mRNA. Notably, *Il7ra* mRNA was more abundant in *Tbx21*$^{-/-}$ ILCs from TRnUC mice in comparison with *Tbx21*$^{+/+}$ ILCs from *Rag2*$^{-/-}$ mice (Figure 61A), consistent with the possibility that T-bet is a transcriptional repressor of the *Il7ra* locus and when absent there is de-regulated/increased expression of *Il7ra*. However, these data should be regarded with some caution as the qPCR data corresponded to a single experiment of flow cytometry sorted ILCs from TRnUC and *Rag2*$^{-/-}$ mice and the data presented in Figure 62 correspond to technical replicates rather than biological replicates and therefore these data should be considered preliminary and in need of replication.

IL-7R expression was also determined at the protein level in ILCs by flow cytometry. Unfractionated splenocytes and cLP cells were labelled with monoclonal antibodies specific for CD90, NKp46 and IL-7Rα. The expression of IL-7Rα was then determined in the CD90^{+} NKp46^{-} population of cells in these organs. In these experiments there was no detectable difference in the expression of IL-7Rα on CD90^{+} NKp46^{-} ILCs in either the spleen or colon of *Rag2*$^{-/-}$ or TRnUC mice (Figure 61B).

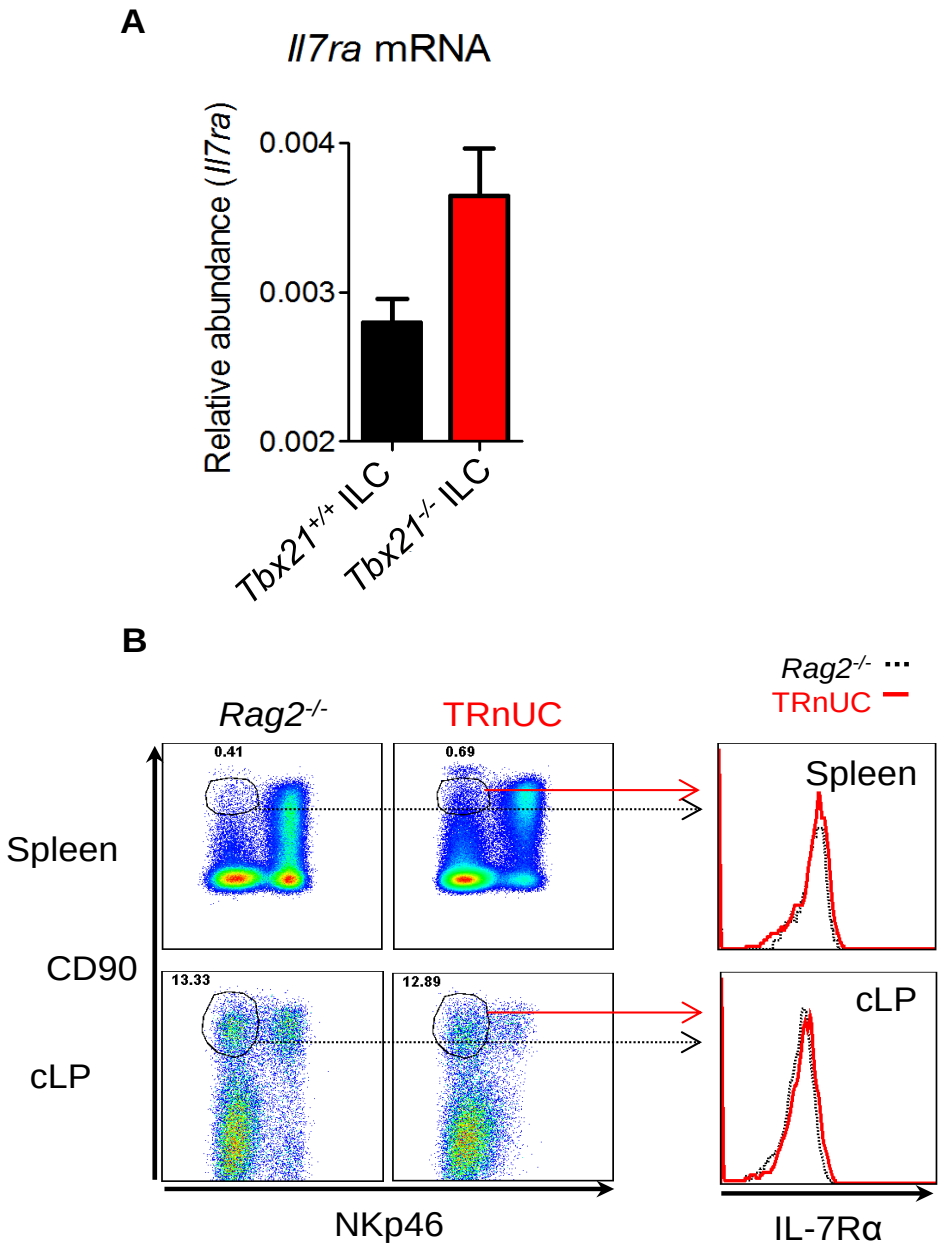


Figure 61. T-bet was a transcriptional repressor of *Il7r*. A qPCR data showing relative expression if *Il7ra* in ILCs from *Rag2*$^{-/-}$ and TRnUC mice following *in vivo* activation with anti-CD40 mAbs. These data represent 2 technical replicates. Bars represent mean relative abundance of *Il7ra* mRNA and error bars denote SEM. Since this graph only represents technical replicates (2 separate analyses) of single ILC populations statistical analysis was inappropriate. In this experiment ILCs were purified by FACS as described in the methods. Cells purity was >95%. B Flow cytometry plot showing the expression of IL-7Rα in ILCs from the spleen and colon of *Rag2*$^{-/-}$ and TRnUC mice. These flow cytometry plots are representative of 4 additional biological replicates. This experiment was repeated on 1 further occasion with comparable results. Adapted from Powell *et al*., 2012.

6.8 Chronic TRUC IBD was dependent on the common γ chain cytokine receptor

IL-7Rα protein expression was not different in ILCs from *Rag2*$^{-/-}$ and TRnUC mice at the time points analysed in these experiments, but it is also possible that T-bet mediated regulation of the *Il7ra* locus operates at different times during ILC ontogeny or under different physiological circumstances. Although additional experiments will endeavour to demonstrate when and under what circumstances T-bet regulates IL-7R protein expression in ILCs, it is also important to demonstrate whether this pathway is relevant in the context of colitis.

The functional IL-7R complex comprises IL-7Rα associated with the common γ-chain cytokine receptor (IL-2Rγ, also known as CD132). In order to determine the functional significance of IL-2Rγ in TRUC disease *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice were generated. It has previously been reported that common γ-chain cytokine receptor deficient mice have >90% reduction in the number of ILCs in secondary lymphoid organs (Kim *et al*., 2005a). The possibility that intestinal ILCs in TRUC disease would also be dependent on this receptor was considered. LP cells were isolated from the colon of TRUC and *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice and stained for CD90 before being analysed by flow cytometry experiments. Triple *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ knockout mice had significantly diminished percentages of CD90^{+} cells in the colon compared to TRUC mice (Figure 62A and 62B). As mentioned previously these data should be interpreted with some caution since comparing the relative proportions of particular cell types can sometimes be misleading as the proportion of an undefined cells type varying between the two genotypes might account for relative shifts in the proportion of other cells. In this experiment ILCs in *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice were barely detectable at all and were diminished 20-fold in comparison with TRUC mice, therefore it is unlikely that the changes observed in ILC proportions were art factual. Indeed, additional subsequent experiments looked at absolute cell numbers (e.g. Figure 62) in a similar context, which gives further support to these observations. There were also important differences in clinical features of *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$

triple knockout mice in comparison with TRUC mice. In contrast to age-matched TRUC mice, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice failed to develop colitis, and had normal (=0) colitis scores (Figure 63A). Similarly, there was no evidence of splenomegaly (Figure 63B), or increased colon weight (Figure 63C) in *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice. In additional to the lack of disease features, the abundance of mRNA encoding *Il17a* was also determined in the colon of TRUC and *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice by qPCR. In contrast to TRUC mice, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice had undetectable levels of mRNA encoding *Il17a* in the colon (Figure 63D).

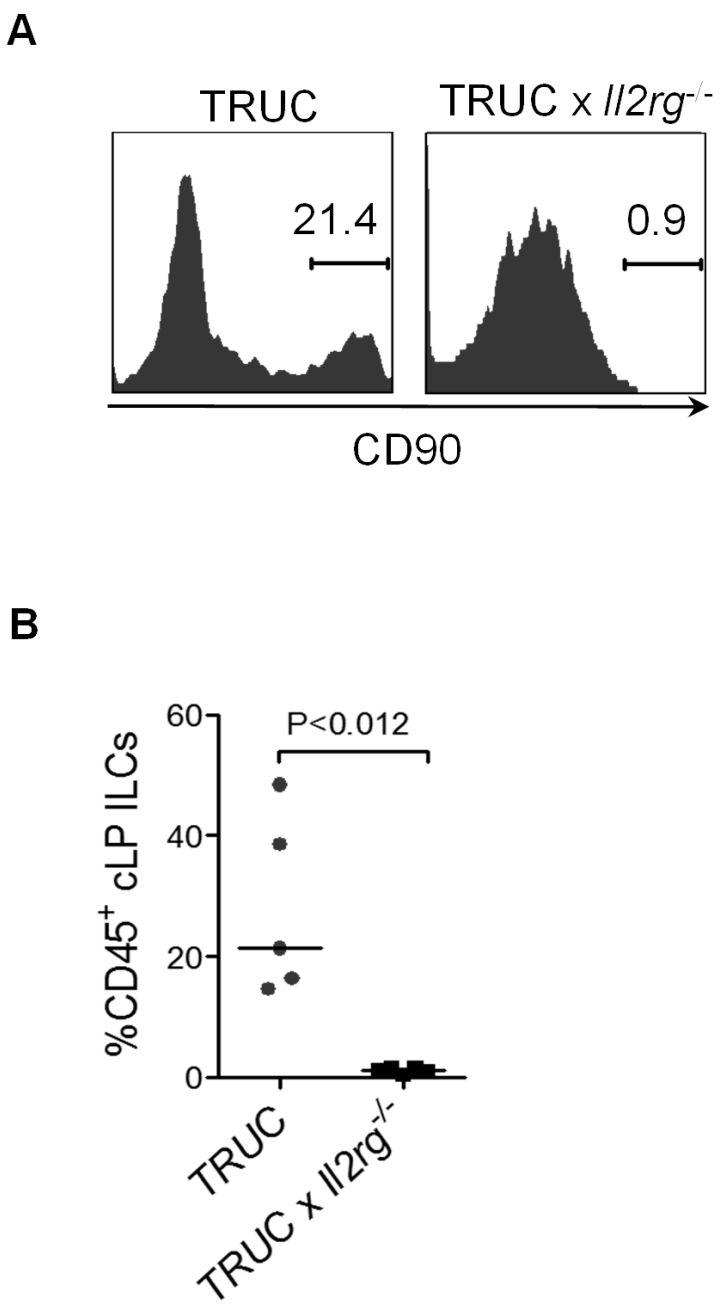


Figure 62. CD90^{high} ILCs were markedly diminished in the colon of *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice. A Flow cytometry histogram showing the % of CD90^{high} ILCs (as a proportion of live, CD45^{+}) in the cLP of TRUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice, and B statistical analysis of age matched biological replicates (n=5 in each group). Each dot represents an individual mouse. The lines depict the median % ILCs among CD45^{+} cells in the cLP. Adapted from Powell *et al*., 2012.

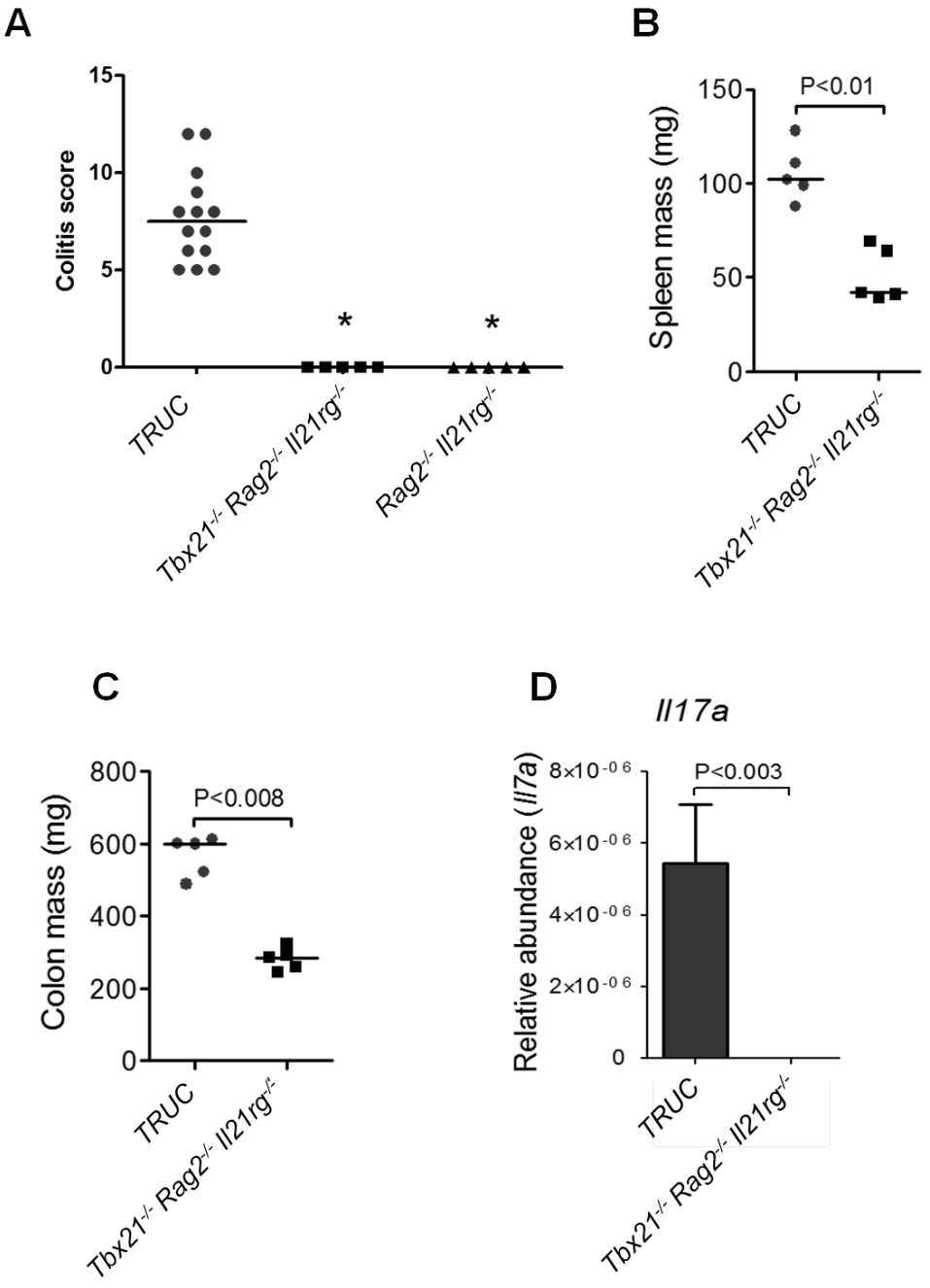


Figure 63. TRUC mice lacking the common γ chain cytokine receptor did not develop colitis. A Colitis score in TRUC (n=14), *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ (n=5) and *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice (n=5). *P<0.001. This experiment was repeated on 2 further occasions with comparable results, but with fewer mice and of different age groups. B Spleen mass and C colon mass in TRUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice. In A-C each dot represents an individual mouse. The lines depict the median of the variable defined by the y-axis). These data are representative data of 2 additional experiments. D Real time PCR of transcripts encoding *Il17a* in the colon of TRUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice. Bars represent mean relative abundance of *Il17a* transcripts and error bars represent SEM. This experiment was a single experiment and no additional experiments have been performed. Adapted from Powell *et al*., 2012.

6.9 *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice gavaged with faeces from TRUC mice failed to develop colitis

Since the microbiota plays such a critical role in TRUC disease, an additional experiment was performed to ensure that *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice were fully exposed to the colitogenic microbiota present in TRUC mice in order to show that the phenotypic differences were due to changes in ILC numbers rather than changes in the composition of the intestinal microbiota. In this experiment TRnUC and *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice were gavaged with a homogenised suspension of faecal bacteria harvested from TRUC mice.

Even following gavage of the colitogenic flora from TRUC mice, CD90^{+} ILCs were barely detectable by flow cytometry in the colon of *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice (Figure 64A and 64B). Similarly, in contrast to TRnUC mice which developed colitis following gavage of homogenised TRUC faeces, *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice failed to develop colitis (Figure 64C).

Notably, inspection of the distal colon of naïve TRnUC mice commonly revealed the presence of lymphoid aggregates within the lamina propria, whereas these aggregates were consistently absent from the colon of *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple knockout mice (Figure 65). Taken together these data are consistent with a non-redundant role for the common γ chain cytokine receptor in TRUC disease.

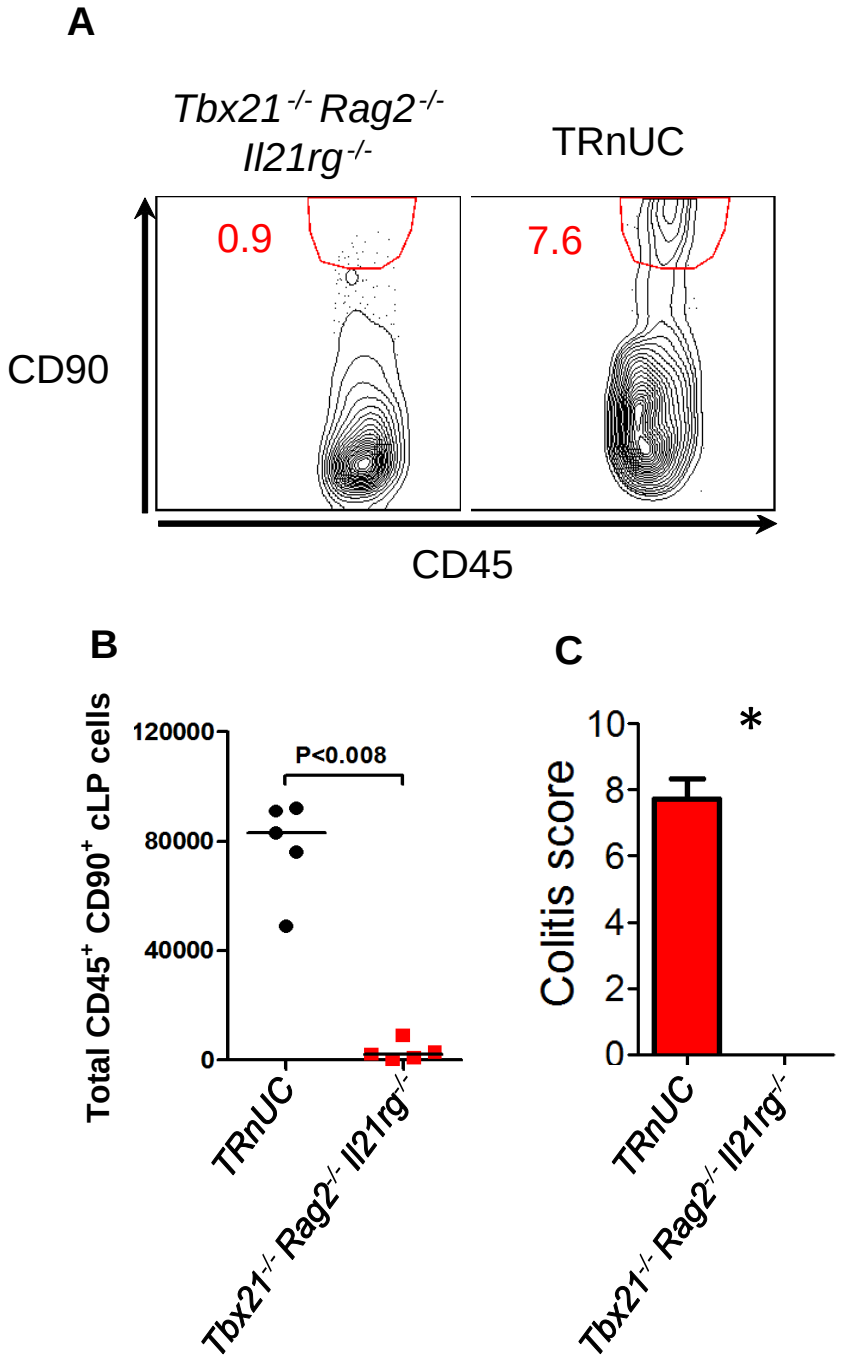


Figure 64. ***Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice gavaged with faeces from TRUC mice were resistant to colitis induction.** A Flow cytometry plot showing CD45^{+} CD90^{+} cells in the cLP of TRnUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice following gavage with faeces from TRUC mice and B total CD45^{+} CD90^{high} cell count. Each dot represents an individual mouse. Lines depict the median total ILC count in the colon. C Colitis score in TRnUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice following gavage with faeces from TRUC mice. Mice were examined 3-4 weeks post-gavage. *P<0.01. Bars denote mean colitis score in the different study groups and the error bars denote SEM. Adapted from Powell *et al.*, 2012.

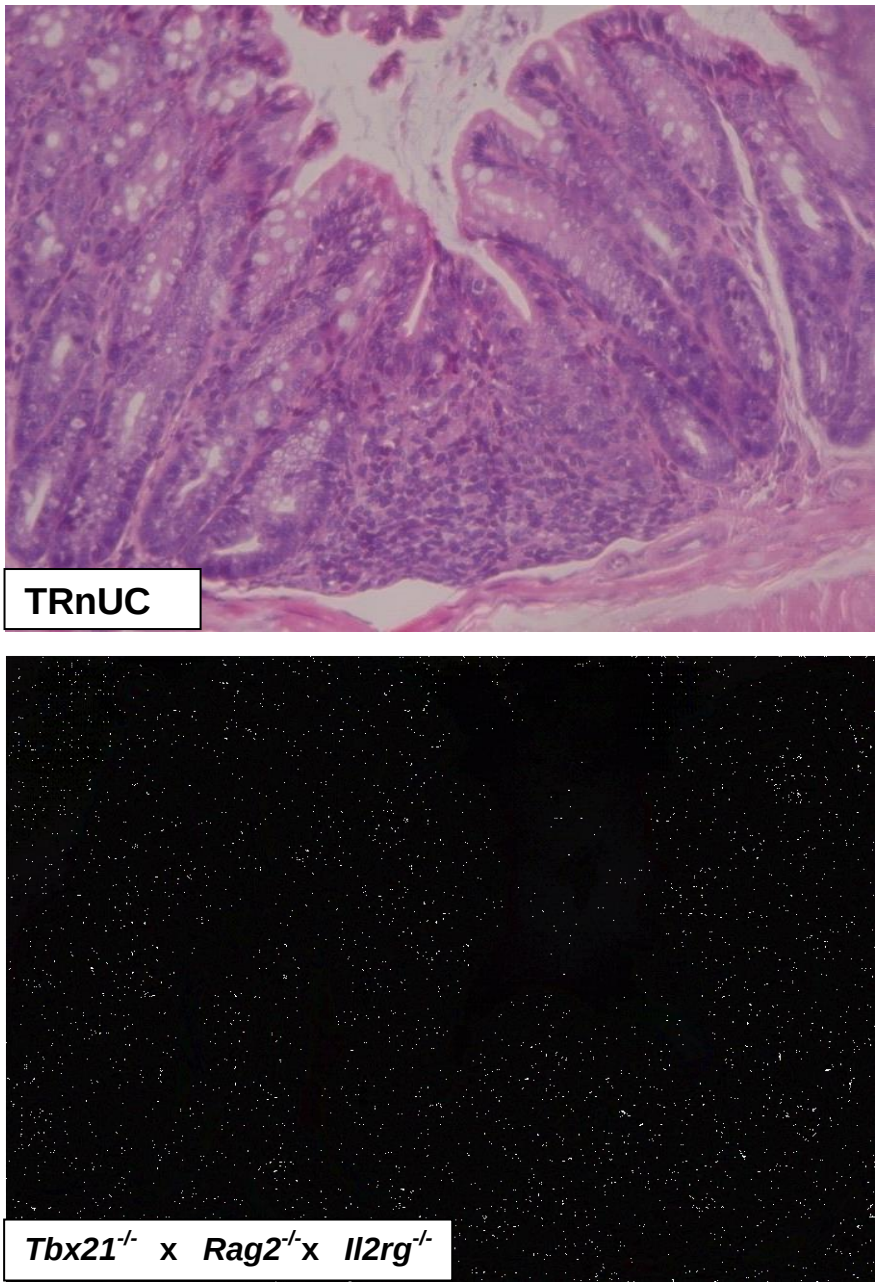


Figure 65. ***Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice lacked lymphoid aggregates in the colon.** Colon micrograph (H&E staining) of the distal colon of TRnUC and *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice. Aggregates of lymphoid cells were commonly seen, even in naïve TRnUC mice, but were consistently absent from the colon of *Tbx21*$^{-/-}$ x *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice (as depicted). Magnification is the same in both panels (approximately x100). These data are representative of multiple analyses.

6.10 TRUC disease was dependent on IL-7 signalling

Although the common γ chain cytokine receptor is essential for IL-7R signalling, it is also a critical component of the receptor complex for other cytokines, including IL-2, IL-4, IL-9, IL-15 and IL-21. Therefore, to dissect the role of IL-7R in TRUC disease antibody mediated blockade of the IL-7R was performed *in vivo*.

Twelve week old TRUC mice were treated with 1mg of anti-IL-7R mAb (n=5, clone A7R34) or control isotype antibody (n=4) administered *i.p.* on days 0, 3, 6, 9, 12, 15 and 18. Mice were culled on day 21 and organs harvested for analysis.

The number of $CD90^+$ ILCs in the spleen and colon of TRUC mice following treatment with anti-IL-7R mAb or control antibody was investigated by flow cytometry. IL-7R blockade significantly reduced $CD45^+$ $CD90^+$ ILC numbers 3-4 fold in the colon of TRUC mice (Figure 66A and 66B). $CD90^+$ $IL\text{-}7R^+$ ILC were also significantly diminished in the spleen of anti-IL-7R treated TRUC mice (Figure 66C and 66D). However, unlike the colon, ILCs were almost completely eliminated from the spleen of anti-IL-7R treated TRUC mice. As discussed previously there is a theoretical risk that using the same depleting mAb as the staining antibody used to identify/show depletion in subsequent flow cytometry experiments might result in lack of detection due to the epitope already being occupied by the depleting mAb which had been administered *in vivo*. In this experiment the same clone was used to block and stain for IL7R (clone A7R34). However, since an additional ILC marker was employed in the staining protocol in this experiment the loss of ILCs was readily apparent not just by loss of cells bearing IL-7R, but by the lack of CD90 cells. In other words if the cells were not being detected because the *in vivo* administered mAb was preventing binding of the staining mAb used in flow experiments then it would be expected that there would be a commensurate increase in $CD90^+$ $IL\text{-}7R^-$ cells in the mice treated with A7R34. However, it is clear from the experiment that CD90 cells are also lost (Figure 66C), thus confirming that blockade of Il-7R results in loss of IL-7R bearing ILCs rather than resulting in an inability to detect them.

IL-7R blockade significantly improved disease parameters in TRUC mice, including a significant reduction in colitis scores (Figure 67A), spleen weight (Figure 67B) and colon weight (Figure 67C), demonstrating that IL-7R signalling plays a functionally

important role in intestinal ILC homeostasis and controls ILC mediated mucosal pathology.

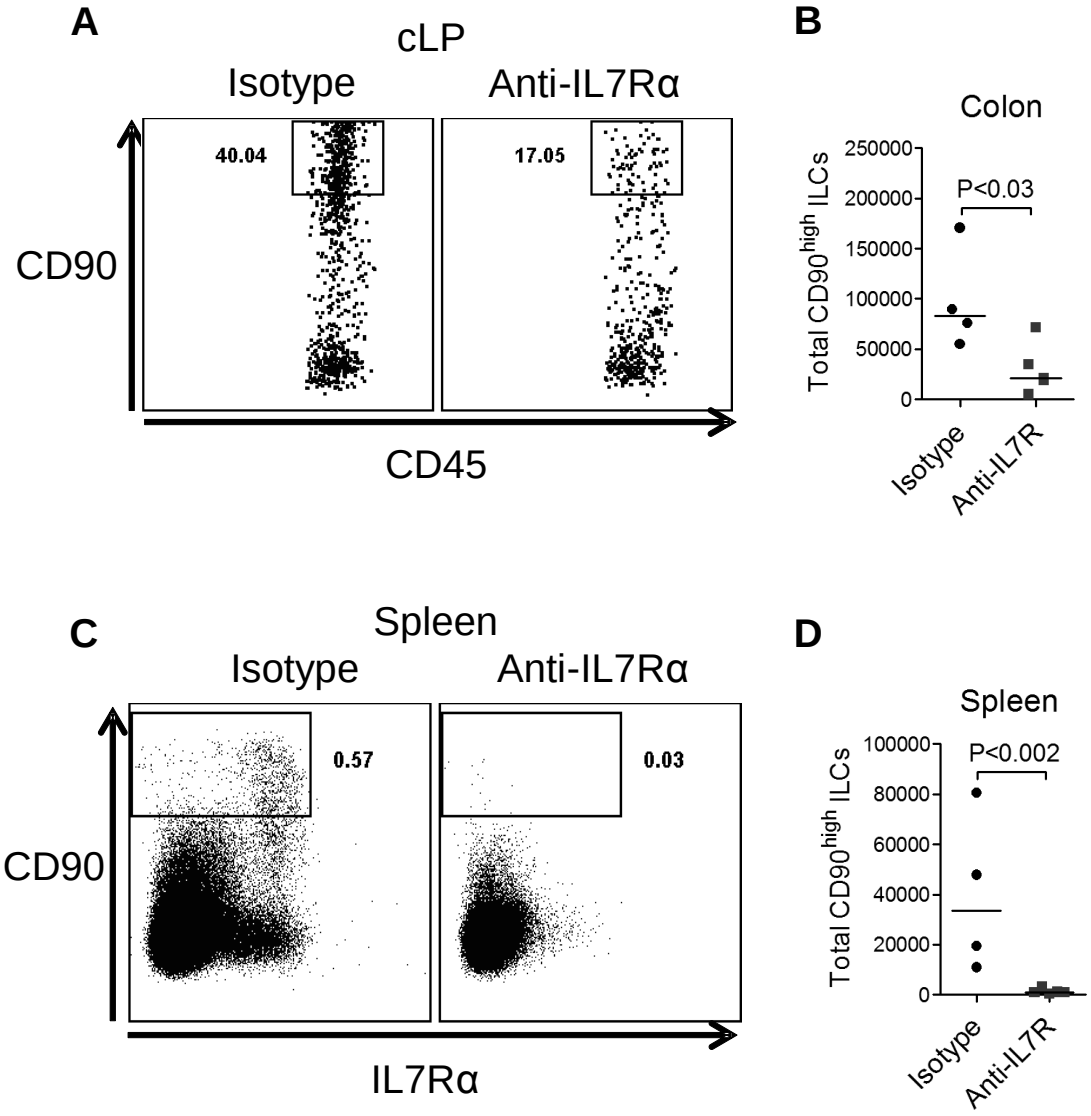


Figure 66. IL-7R blockade reduced the number of ILCs in the colon and spleen of TRUC mice. A Flow cytometry plot showing $CD45^+$ $CD90^+$ cells and B absolute number of $CD90^{high}$ ILCs in the colon of TRUC mice treated with anti-IL-7R mAb or control (isotype) antibody. C Flow cytometry plot showing $CD90^+$ $IL\text{-}7R^+$ cells and D absolute number of $CD90^{high}$ ILCs in the colon of TRUC mice treated with anti-IL-7R mAb or control (isotype) antibody. The flow data illustrated are representative of 4 biological replicates in each group. Insufficient cells were available for analysis from 2 mice (one in each group). This *in vivo* blocking experiment was only performed once. In Figures B and D each dot represents an individual mouse. Lines depict median of parameter defined by the y-axis. Adapted from Powell *et al*., 2012.

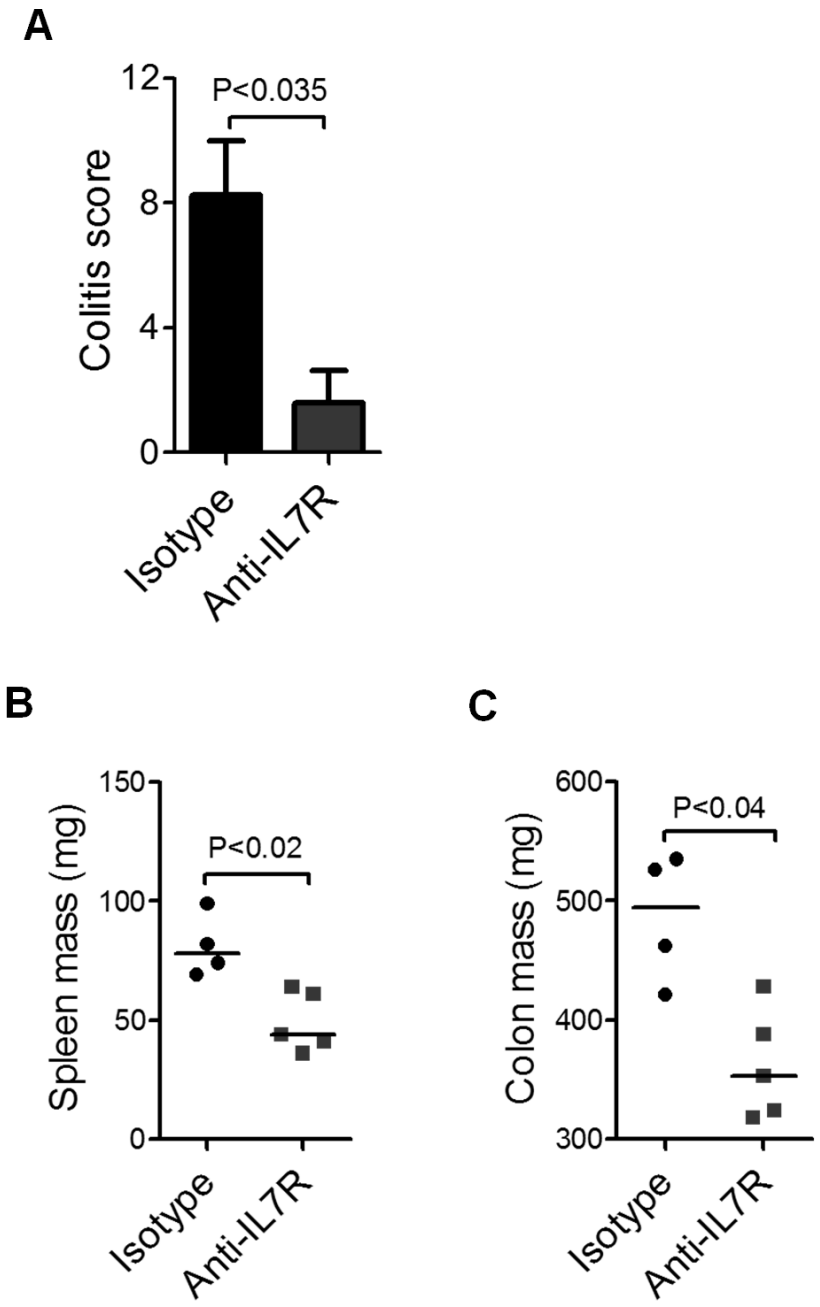


Figure 67. IL-7R blockade attenuated TRUC disease. A Colitis histology scores, B spleen mass and C colon mass of TRUC mice treated with anti-IL-7R mAb or control (isotype) antibody. There were 5 mice in each group. In panel A the bar represents the median histology score in the study group and the error bars represent SEM. This *in vivo* blocking experiment was only performed once. In panel B and C each dot represents an individual mouse and the lines represent the median (of the parameter defined by the y-axis). Adapted from Powell *et al*., 2012.

6.11 Discussion

Some of the specific molecular mechanisms that impact on colitis development as a consequence of T-bet deficiency have been defined. TRUC mice are prolific producers of cytokines, such as IL-17A and IL-22, yet are relatively poor producers of IFNγ. Indeed, in microbiota-dependent (HT-induced) or microbiota-independent (agonistic anti-CD40 induced) models of intestinal inflammation, intestinal ILCs from T-bet sufficient *Rag2*$^{-/-}$ mice predominantly produce IFNγ, whereas T-bet deficient ILCs from TRnUC mice predominantly produce IL-17A, with relatively few IFNγ producing cells. These data indicate that T-bet is necessary for optimal IFNγ expression by intestinal ILCs. These data are consistent with studies in T-cells in which T-bet is required for transactivation of the *Ifng* locus (Szabo *et al*., 2000). It therefore seems most likely that T-bet is also required for optimal transcription of the *Ifng* gene in ILCs as well as in T-cells. Although, T-bet has been shown to bind at the *Ifng* locus in CD4^{+} T-cells, these experiments are technically challenging with ILCs since many millions of cells are needed to perform ChIP experiments and it is very difficult to isolate this many ILCs from the colon of TRUC mice, even if cells are pooled from different donors. Loss of IFNγ production and paradoxical augmentation of IL-17A production by T-bet deficient ILCs mirrors observations in T-bet deficient CD4^{+} T-cells in which T-bet also appears to be a repressor of IL-17 production (Durrant *et al*., 2009; Harrington *et al*., 2005; Park *et al*., 2005; Gokmen *et al*., 2012). Given the enhanced IL-17A production in the absence of T-bet in t-cells and ILCs it is possible that the molecular mechanisms responsible for this phenotype are conserved across these particular lymphoid lineages. Possible mechanisms include loss of T-bet mediated suppression of IL-23R expression (Harrington *et al*., 2005), loss of T-bet mediated limitation of Runx1 transactivation of *Rorgt* (Lazarevic *et al*., 2011), or loss of T-bet mediated repression of IRF4 (Gokmen *et al*., 2012). Diminished innate production of IFNγ observed in T-bet deficient hosts likely accounts for the delayed wasting disease observed in anti-CD40 induced disease, consistent with previous reports showing that systemic disease in this model is mediated by the IL-12/IFNγ pathway (Uhlig *et al*., 2006). These data also imply that T-bet impacts on innate immunity in a tissue specific manner, and that intestinal rather than systemic disease is favoured in its absence. In contrast to other studies

showing that ILC-mediated intestinal pathology requires dual blockade of IL-17 and IFNγ to abrogate disease (Buonocore *et al*., 2010), in TRUC IBD, IL-17A blockade alone was sufficient to reverse disease. It is possible that IL-17A blockade alone would not fully prevent ILC mediated pathology in T-bet sufficient hosts because unlike TRUC mice these animals can still mount effective IFNγ responses. These data are also consistent with recent hypotheses suggesting that ILCs form distinct functional lineages, similar to effector T-cell populations. T-bet appears to be an important molecule responsible for regulating the profile of cytokines produced by ILCs. In the absence of T-bet these cells fail to mount a significant IFNγ response, and instead preferentially produce IL-17A.

In this study signalling through the common γ-chain cytokine receptor (*Il2rg*) was shown for the first time to be functionally important and indispensable in TRUC disease. *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple deficient mice were resistant to colitis development even following exposure to the colitogenic flora present in TRUC mice. The common γ-chain cytokine receptor is a component of multiple different cytokine receptors including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Yamane and Paul, 2012), and therefore deficiency of this receptor could potentially impact numerous cell types, including macrophages, dendritic cells , NK cells as well as ILCs. It might be argued that loss of NK cells in *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ triple deficient mice might account for the impaired inflammatory response observed in the colon of these mice, since NK cell development is totally dependent on IL-15 signalling, and its receptor belongs to the common γ-chain cytokine receptor family (Vosshenrich *et al*., 2005). However, other evidence makes this unlikely. Firstly, T-bet plays an important role in NK cell ontogeny/maturation and consequently mice lacking T-bet have markedly diminished numbers of NK cells (Townsend *et al*., 2004; Soderquest *et al*,. 2011). Therefore, it seems improbable that NK cells are playing a significant pathological role in TRUC disease as these cells are markedly diminished in these mice by virtue of their T-bet deficiency. Furthermore, in other models of colitis NK cells appear to play a protective or regulatory role rather than an inflammatory role. For instance, depletion of NK cells in *Il10*$^{-/-}$ mice, which develop spontaneous colitis, results in even more severe disease (Fort *et al*., 1998). Therefore, it seems unlikely that loss of NK cells due to loss of IL-15 signalling would confer resistance to TRUC colitis as

was observed in *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice. In this study it was shown that the cytokines IL-4 and IL-21 are unlikely to play a significant role in TRUC disease since these cytokines are either undetectable or at low levels comparable to disease free *Rag2*$^{-/-}$ mice, therefore, it is unlikely that these common γ-chain cytokine receptor family members play a significant role in the resistance of *Tbx21*$^{-/-}$ *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice to colitis induction. IL-9 is predominantly expressed by T-cells and plays an important role in allergic responses (Stassen *et al*., 2012), and its expression in the colon of TRUC mice was not specifically analysed in this study. Although in this study it was clearly demonstrated that ILC numbers are reduced in the context of common γ-chain cytokine receptor deficiency, it would be beneficial to determine the impact of losing this receptor on other key innate immune cells in the colon of TRUC mice.

However, evidence for an important role for another common γ-chain cytokine receptor, IL7R, was seen in further *in vivo* blockade experiments in TRUC mice. Blockade of the IL-7R with specific mAbs resulted in a significant reduction in ILC numbers (particularly in the spleen and to a lesser, but significant degree in the colon) in TRUC mice. This was associated with reduced innate IL-17A expression and significantly attenuated disease in TRUC mice. Interestingly, unlike splenic ILCs that were almost completely eliminated, colonic ILCs were less prone to depletion by IL-7R blockade, indicating that additional IL-7 independent ILC survival signals may exist in the colon or in inflamed tissue.

Further evidence for an important role for IL-7R in TRUC disease (as a consequence of T-bet deficiency) came from molecular studies. Crucially, T-bet binds at the *Il7ra* locus (at least in CD4 T-cells), consistent with the likelihood that T-bet transcriptionally regulates IL-7R expression and thereby may control ILC homeostasis. Indeed, T-bet deficient ILCs had increased expression of mRNA encoding *Il7ra*, indicating that T-bet is a transcriptional repressor of this locus in ILCs. However, it should be noted that these experiments should be considered preliminary, as the data presented in this thesis are from a single experiment (with technical replicates). Although the data support the hypothesis that T-bet is a transcriptional repressor of the *Il7ra* locus these data will need replicating in future work. The importance of IL-7R signalling in human IBD is also implied by recent

data identifying single nucleotide polymorphisms at the *IL7R* locus in patients with UC (Anderson *et al*., 2011).

In conclusion, potential molecular mechanisms whereby T-bet controls ILC phenotype in TRUC disease include regulation of cytokine production (IL-17A versus IFNγ) and possibly through regulation of IL-7R expression.

CHAPTER 7

Conclusions

7.1 Updating the TRUC paradigm of intestinal inflammation

The data presented in this study along with prior work allows the TRUC paradigm of colitis to be expanded (Figure 68) and also allows further parallels with human IBD to be drawn. The earliest abnormality present in TRUC mice is increased mucosal TNFα-induced colonic epithelial apoptosis which subsequently results in impaired intestinal barrier function (Garrett *et al*., 2007). The data presented in this thesis data corroborate these observations and confirm that TNFα expression is constitutively increased in T-bet deficient DCs, which is even further enhanced following exposure to "colitogenic" microbes, such as *Helicobacter typhlonius*. Data in this thesis show for the first time that $CD11b^{+}$ $CD103^{-}$ are both the most populous and chief source of TNFα in the colon of TRUC mice. These data are consistent with reports from other groups suggesting that DCs characterized by expression of CD11b and CX_3CR1, but lacking CD103 are the major inflammatory subset of gut dwelling DCs in mice in the context of intestinal inflammation (Varol *et al*., 2009).

Excess TNFα production in TRUC disease is responsible for driving increased colonic epithelial apoptosis and impaired barrier function, the earliest lesions observed in TRUC mice, predating overt colitis (Garrett *et al*., 2007). Impaired epithelial barrier function is also implicated in human inflammatory bowel disease. Polymorphisms at loci encoding genes involved in maintaining the intestinal barrier, such as *ECM1*, *CDH1*, *HNF4a* and *laminin B1* have been identified as susceptibility loci in UC (Thompson and Lees, 2011). Increased intestinal permeability has also been observed in subgroups of healthy first degree relatives of IBD patients (Hilsden *et al*., 1996; Peeters *et al*., 1997), suggesting that epithelial abnormalities may be associated with IBD risk, but by themselves are insufficient to instigate disease without an additional environmental cue.

Chronic TRUC disease was dependent on IL-17A secreting $CD90^{+}$ ILCs. As well as attenuating disease, blockade of IL-17A, the chief effector cytokine produced by

ILCs in TRUC mice, markedly reduced colonic neutrophil recruitment, which is a key feature of chronic TRUC disease. The absence of T-bet in TRUC mice resulted in preferential production of IL-17A at the expense of IFNγ. In T-bet sufficient *Rag2*$^{-/-}$ mice, ILCs expressed T-bet which correlated with IFNγ production. However, T-bet deficient ILCs were poor producers of IFNγ, and instead preferentially produced IL-17A, resembling the phenotype of *Tbx21*$^{-/-}$ T-cells (Lazarevic et al., 2011).

In this thesis is has been shown that key cytokine signals responsible for driving ILC activation are IL-23 and IL-6. Furthermore, TNFα was observed to synergise with IL-23 to stimulate IL-17A. The TNF superfamily member TL1A was also found to be increased in TRUC disease, and was observed to weakly promote IL-17A production as well. TL1A also seemed to synergise with IL-23 to promote IL-17A production in TRUC mice. However, unlike IL-23 and IL-6, which were shown to be functionally important activators of innate immunity in the gut, since blockade of these cytokines attenuated TRUC disease, neutralization of TL1A failed to control TRUC disease. However, it was discussed that these blocking experiments were likely suboptimal therefore the functional role of TL1A in TRUC should be considered yet to be conclusively defined and a clear role for TL1A in driving innate immune mediated colitis remains to be demonstrated.

TRUC disease is characterised by chronic, progressive colitis with the severity of disease increasing with time (Garrett *et al*., 2007; Garrett *et al*., 2009; Powell *et al*., 2012). Accordingly, additional studies will focus on the contribution of different immune cells over time. Since early TNFα production triggers epithelial apoptosis in the colon of TRUC mice as young as 2-4 weeks old (Garrett *et al*., 2007) the cellular sources of this cytokine should probably be considered the most proximal cellular mediators of disease. In the initial studies TNFα production predominantly mapped to CD11c DCs, although a contribution from macrophages cannot easily be discounted in view of some shared cell surface phenotypic markers between these related myeloid cells. It is likely that DC derived TNFα is very important in these early events, which rapidly leads to early loss of epithelial integrity. Indeed, early rather than late blockade of this cytokine is therapeutically beneficial in TRUC mice and TNFα blockade in TRUC mice aged >12 weeks old lacks efficacy, indicating that other cytokine networks and alternative cells emerge as important mediators. It

is tempting to speculate that ILCs (perhaps driven by IL-23, IL-6 or TL1A) are responsible for chronic TRUC disease, especially once the effectiveness of the colonic epithelial barrier has been compromised and inflammatory cascades are initiated by the innate immune compartment. However, mice lacking ILCs from birth (e.g. TRUC x *Il2rg*$^{-/-}$ mice) are totally resistant to colitis, consistent with the possibility that ILCs are also involved in early disease. However, given the widespread expression of the common γ–chain receptor across many immune cells it is difficult to discount a contribution from other cells types in these early phases of disease as well. Undoubtedly ILCs play an important in the chronic phase of disease, since depletion of these cells, even in late disease (mice >12 weeks old) is curative. Future experiments will focus specifically on the role of ILCs in early TRUC disease versus late TRUC disease. The role of macrophages and NK cells in early and late disease in TRUC mice also merits research attention in the future.

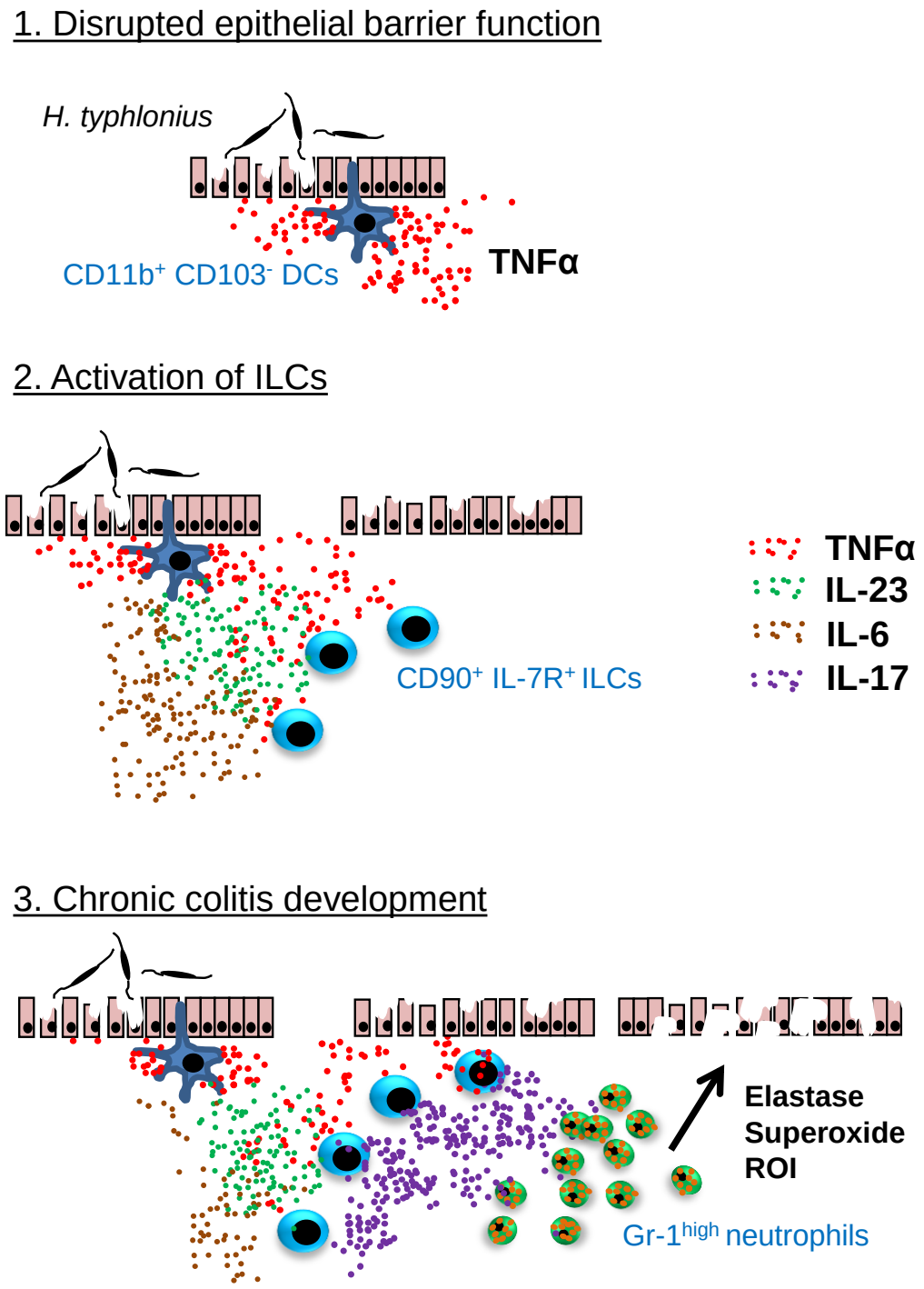


Figure 68. The updated TRUC paradigm of IBD. Particular constituents of the intestinal microbiota (such as HT) drive excess TNFα production by colonic $CD11b^+$ $CD103^-$ DCs, leading to epithelial apoptosis and loss of epithelial barrier function. This triggers cytokine signals, including IL-23, IL-6 and TNFα, which drive IL-17A production by $CD90^+$ $IL\text{-}7R^+$ ILCs, which in turn leads to neutrophil recruitment and propagation of chronic intestinal inflammation, with further epithelial damage from neutrophil products, such as reactive oxygen intermediates (ROI) and elastase.

7.2 Translating lessons from new insights into TRUC disease in to clinical practice

The TRUC model of IBD provides some interesting insights into current, experimental and future therapies in IBD. IBD therapies are unsatisfactory. For instance, after one year of treatment with azathioprine, the first line treatment for CD, only about a quarter of patients will still be in remission (Colombel et al., 2010). This has prompted pharmaceutical companies to search for newer "biological" targets. The first of these to emerge was infliximab, a humanised mouse mAb that specifically targets TNFα. Infliximab and newer, fully humanised mAbs targeting TNFα are now commonly used to treat both CD and UC. However, infliximab monotherapy will only keep a third of CD patients in remission after 1 year of treatment (Colombel et al., 2010), and even optimal therapy with a combination of infliximab and azathioprine manages to maintain remission in less than half of CD patients after 1 year of treatment (Colombel et al., 2010). It is notable that anti-TNFα therapies are also effective in early TRUC disease. TNFα synergised with IL-23 to augment IL-17A production by ILCs which could be significantly attenuated by TNFα blockade, highlighting a previously unrecognised potential therapeutic action of anti-TNFα antibodies. It is interesting that TNFα blockade is most effective in early TRUC disease (Garrett *et al*., 2007; Garrett *et al*., 2009), and although many factors are likely involved, in human IBD the clinical response to anti-TNFα agents are also greatest in patients treated early in their disease course, and are less effective in patients with firmly established disease (Schreiber *et al*., 2010).

Similarly, IL-23 is identified as a key driver of ILC effector function in TRUC disease and for the first time it is shown that antibody mediated blockade of IL-23p19 attenuated chronic TRUC colitis and limited innate IL-17A production. These data are consistent with the possibility that blockade of IL-23 mediated ILC activation maybe one of the therapeutic mechanisms of action of anti-IL-12p40 mAbs that have shown some promise in IBD (Mannon *et al*., 2004). These data also lend credence to the development of specific anti-IL-23p19 therapies as possible future therapies for IBD.

In this work it was shown that IL-6 is elevated in TRUC disease and when blocked *in vivo* results in reduced innate production of IL-17A and attenuated TRUC disease.

Biological therapies targeting IL-6 or its receptor, such as tocilizumab, are efficacious in the treatment of other inflammatory diseases, including arthritis (Smolen *et al*., 2008; Yokota *et al*., 2008) and lupus (Illei *et al*., 2010). A pilot study of anti-IL-6R therapy in Crohn's disease had promising initial results (Ito *et al*., 2004), and awaits validation in a large, randomised placebo-controlled trial. Currently, there are no data on blocking the IL-6 pathway in UC.

This thesis also potentially provides some novel insights in to therapies blocking IL-17A and IFNγ. In contrast to other studies showing that ILC mediated intestinal pathology requires dual blockade of IL-17 and IFNγ to abrogate disease (Buonocore *et al*., 2010), in TRUC IBD IL-17A blockade alone reversed disease. It is possible that IL-17A blockade alone would not fully prevent ILC mediated pathology in T-bet sufficient hosts because unlike TRUC mice these animals can still mount effective IFNγ responses. In other words, since ILCs from T-bet deficient hosts are unable to mount an effective IFNγ response, then blockade of IL-17A alone was sufficient to cure chronic IBD in TRUC mice. These observations offer potentially relevant insights into clinical trials evaluating blockade of these cytokines in human IBD. Antibody blockade of IFNγ is largely disappointing in Crohn's disease (Reinisch *et al*., 2010) and recently a placebo controlled trial of an anti-IL-17A mAb (secukinumab) showed that blockade of this cytokine therapy lacked efficacy, or even exacerbated moderate to severe CD (Hueber et al., 2012). However, in both of these studies improvements were seen in certain patient sub-groups, including those with raised inflammatory markers (anti-IFNγ) or in patients with particular genetic polymorphisms (risk variant alleles at the *TL1A* locus in anti-IL-17A treated patients).

Redundancy and plasticity of IL-17A and IFNγ responses also potentially accounts for the lack of efficacy observed following monotherapy with either anti-IL-17 or anti-IFNγ. Trials evaluating dual blockade of these cytokines, and thereby limiting plasticity/redundancy might prove to be useful in these difficult diseases. Alternatively, blockade of IL-12p40, which is required for biological activity of both IL-12 and IL-23, and hence is a common upstream signal for both IL-17A and IFNγ production, is efficacious in CD (Mannon *et al*., 2004). In the future it is conceivable that IBD patients will be stratified according to their genetic profile or their mucosal immune response in order to better guide selective cytokine blockade in individual patients.

7.3 ILCs and the genetic risk of IBD

The data presented in this thesis also potentially provide novel interpretations of current understanding of the genetic basis of IBD. At present there are 163 genes now associated with altered risk of developing IBD (Jostins *et al*., 2012). Most of these are common to both CD and UC. Although innate pathways, such as NOD2 and autophagy are well recognised, particularly in CD, it has been assumed that a large number of risk variant alleles are implicated in altering the phenotype of adaptive immune cells, and in particular, effector T-cell lineages, such as Th1 and Th17 cells function (Jostins *et al*., 2012). However, one could also make the case that most of these risk alleles might equally impact ILC phenotype. Among these alleles include IL-23/IL-17 axis or the IL-12/IFNγ axis molecules, such as *IL12A* (IL-12p40 subunit common to both IL-12 and IL-23), *CCR6, IL23R, IFNG, RORC and STAT3*, all of which are now known to be expressed by or are involved in the regulation of gut ILCs. Similarly, polymorphisms at loci encoding *IL7R, TL1A, IL6ST* and *CD25* are also associated with IBD (Jostins *et al*., 2012), and are likely involved in ILC biology as well as T-cell biology. Now that ILCs have been identified in inflammatory lesions of IBD patients, the impact of risk variant polymorphisms in ILC phenotype may yield new insights into these important diseases.

7.4 Future work

In addition to increased expression of IL-17A, it was also noteworthy that TRUC disease was also characterised by increased expression of IL-22. Further work will focus on the role of this important cytokine in TRUC disease. There is also a pressing need to translate these crucial pre-clinical findings to human disease. Indeed, preliminary studies show that ILCs are present in the colon of UC patients and that IL-23 and IL-6 are capable of stimulating these cells to produce IL-17A.

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